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EDITORIAL

TUBERCULOSIS

In the report on the Animal Health Services in Great Britain for 1955 it is stated: "The rate of attestation has been greatly accelerated during the last few years . . . there is every reason to hope that by the early 1960's bovine tuberculosis in Great Britain will, for all practical purposes, be a thing of the past." Tuberculosis is a disease which has affected man and animals during all the centuries of which there is record. Bones of the palæolithic and neolithic periods have been found to show evidence of tuberculosis. The disease may be older than man and it would not be surprising if the bones of some dinosaur were to yield evidence of its ravages. It was certainly prevalent in ancient Egypt, for lesions of tuberculosis have been found in the bones of mummies of the Rameses period thirteen centuries B.C.

Even earlier record is available, for the Hindu Veda of the fifteenth century B.C. referred to the disease. The Greeks knew of it. Hippocrates observed it in Greece in the fifth century B.C. and Aretacus noted its occurrence in Asia Minor in the first century B.C. The Jewish Talmud of the first century A.D. prohibits the consumption of affected flesh and the Mishna (third century A.D.) and the Gemara (fifth century A.D.) support this prohibition.

In the fourteenth and sixteenth centuries similar laws were made in Germany. In 1702 the Germans seemed to think that it was identical with Syphilis, the so-called "French Disease," and it was ordered that all visibly affected cattle were

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XUM

to be slaughtered. In 1783 two types of the disease were recognised, human and animal, and, no doubt as a result of that accord, it was assumed that the disease was not transferable from one species to another and the laws relating to meat inspection were waived completely.

In those days the germ theory of disease was unknown, but it is interesting to ponder on what might have been the ultimate effect on the incidence of the disease if the original regulations relating to compulsory slaughter of all visibly affected animals had not been abandoned.

Until about 100 years ago men had to depend entirely upon clinical observation and philosophical deduction in their attempts to resolve the problems of disease. Many acute observors were interested in tuberculosis. Rushling (Germany, 1774) and Huzard (France, 1790) recognised its contagious nature. Huzard thought that tuberculosis of man and animals was identical, and this view was supported at a later date by Gurlt (1831) and Spinola (1858). In England, Reid (1782) and Baille (1793) drew attention to the fact that tubercles found in the chest grew in size and gave rise to lung abscesses.

Laènnec (1781-1826), the distinguished French physician who introduced the stethoscope and used the ear as an aid to diagnosis, was himself a victim of tuberculosis. He paid great attention to the disease. He considered that it was communicable and was caused most probably by some specific agent or organism. In 1865 Villemin recorded the transfer of human and animal tuberculosis to rabbits and guinea-pigs, and his findings were confirmed by workers in England, France and Germany. Pasteur (1822-1895) and Robert Koch (1843-1910) then came on the scene. Pasteur enunciated the germ theory of disease and Koch discovered the tubercle bacillus in 1882. He recovered it from lesions in men and animals. He was able to isolate it, grew it in pure culture, reproduced the disease in healthy animals and recovered the organism again.

In 1889 Rivota and in 1890 Maffuci demonstrated that the organism which caused tuberculosis in birds was slightly different from that which caused the disease in mammals, and in 1896 Theobald Smith (1859-1934) differentiated human from bovine bacilli. After these discoveries three types of bacilli were recognised: human, bovine and avian.

At the turn of the present century the possible infection of an animal of one species by the type of organism which was responsible for the disease in one of another species was not accepted by all workers. The work of the Royal Commission on Tuberculosis and particularly that of our own great man, Sir John McFadyean, made it abundantly clear that bovine tubercle bacilli could infect man and propagate in his body. It may seem strange to those of the modern generation that there could have been any divergence of opinion amongst the great men who conducted research on this disease, but we must remember that they were sailing on an uncharted sea and as a result of their labours the task of the modern worker has been eased for he has been seeded many hurdles in the race for information. We must remember also that some of his illustrious contemporaries did not accept Harvey's account of the circulation of the blood.

After the report of the Royal Commission the potential danger of the bovine bacillus to man and especially to children was recognised by most people. A minority did not accept the idea, and indeed in 1957 there are some who refuse to conform. This lack of unanimity is not a bad thing. If a man employs two advisers in his business and they are always in agreement the employer can dispense with the services of one of them without hurt. All schools of thought do agree that it would be good if tuberculosis could be eliminated from or rendered harmless in both species, man and animal.

This object can be sponsored in two ways. One is by attempting to protect by vaccination and the other by controlling all potential centres of infection by hygienic measures, which include isolation and in the case of animals may justify slaughter. Hygienic measures plus vaccination have been used in the case of human victims and the slaughter method of elimination has been applied to cattle. Both methods have registered considerable success. We do know that the body responds actively to the stimulus of infection and recovery from an infectious disease depends greatly upon the degree of resistance which is provoked. There has been a great reduction in the incidence of human tuberculosis during the last 50 years. Sanatoria for the treatment of tuberculosis have beds to spare, some have closed for want of patients, and it may be that the end of the human scourge is in sight.

It is possible that even if nothing had been done the incidence and severity of the disease in man might have been greatly reduced in the natural way that all infectious diseases follow, but the time factor would have been extended and the toll of life and suffering much greater. In cattle, as the disease can be measured by an economic standard and sentiment be discounted, the slaughter policy, which was first introduced by J. B. Simonds when cattle plague swept the country in the 19th century, has been the method employed. When an efficient tuberculin had been devised the attack on tuberculosis in cattle went into full gear. In less than twenty years the Ministry of Agriculture has been able to make the momentous statement quoted above. If the prophesy is fulfilled and if human tuberculosis also comes to an end in the near future, all arguments will become purely of historic academic interest.

XUM

GENERAL ARTICLES

ON THE ROUTINE CHEMICAL ANALYSIS OF SMALL VOLUMES OF URINE

Part 1—Review of Methods

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Introduction

THE chemical analysis of urine is an important aid in clinical diagnosis. Most of the tests are well known, but it is doubtful if they are always carried out to the best advantage or in the most economical manner. The majority of the older tests for abnormal constituents require the use of relatively large volumes of urine, 5 ml. or more, and reagents in solution. Obviously a routine test that employs a " drop " of urine, and a correspondingly small quantity of the dry test reagent either in a small tube or impregnated in a test paper will have distinct advantages. Many simplified tests have been devised for use in human medicine, and most of these appear to be as sensitive and specific as their older precursors. There is, of course, every reason to employ similar tests in veterinary medicine. On the whole all vertebrate urines are similar, as also are the abnormal constituents that arise as the result of stress and disease. A further argument in favour of the " drop " technique is that small domestic and laboratory animals often cannot supply more than a very few ml. of urine in a single voiding—a situation that arises rarely in human medicine or in a large animal practice. There is practically no limit to the modifications that can be made to the classical tests in order to meet any special requirements. Most of these are fairly simple and require only a little ingenuity. Usually the ultimate result is a saving of time, labour and equipment. All the tests that are described in this communication are in common use in some form or another. Any of the modifications suggested are not intended to be final, but merely to stimulate interest and development in this often-neglected branch of veterinary medicine.

Range of tests, and a comment on quantities.

Tests are described for the determination of specific gravity, *pH*, for the detection, estimation and differentiation of glucose lactose and some other common reducing substances, for the detection of protein, ketones (acetone and acetoacetic acid), blood and bile pigments. The identification of exotic and more rarely occurring abnormalities is not considered at this stage. Continuous use is made of the word " drop." Although this vague description of measurement is not entirely desirable, it is considered that it is justified in this scheme, but only if the user always maintains the same drop size in all tests. As a general rule the drop referred to in this communication is the smallest volume of liquid that is delivered from an ordinary or bacteriological type 1 ml. pipette. This is about 0.02 ml. A small Pasteur pipette, or fountain-pen filler, will deliver a

similar volume of liquid. The author has found that a 1 ml. tuberculin syringe graduated in 0.001 ml. divisions and fitted with a No. 20 needle is an excellent substitute pipette for use in quantitative routine analysis. Equipment and chemicals that are required are all fairly cheap and readily obtainable commercially. They are set out under the individual tests, as also are the number of tests that can be carried out with the stock quantity of reagent recommended. In all cases tests should be carried out on fresh urine. If it is necessary to add a preservative, a small crystal of thymol is recommended. The sample should be kept as cool as possible.

The tests described will permit a full routine analysis of eight or nine tests to be made on about 0.2 ml. of urine. A small "field" testing set embodying some of the described reactions is now being developed.

Standards.

In carrying out any test, four general rules should be remembered:—

First: Compare the unknown with a "blank."

Second: Compare the unknown with a standard.

Third: Repeat every test.

Fourth: Ensure a continuity of method.

Probably the most difficult rule to apply continuously is the second. Provision of suitable standards is always difficult in a surgery, and there is the disadvantage that long storage of biological fluids causes their deterioration. This gives false results. Coffin (1953) recommends the use of plasma for carrying out some tests and checking reagents. This is not difficult to obtain fresh when required. Bile can also be obtained freshly. For the remainder, glucose, lactose and acetone should be kept undiluted. Sugars can be kept in 1 g. or 2 g. quantities in small stoppered tubes.

Protein standards are probably the most difficult to obtain, but reference to some normal values will enable standards to be made that are unlikely to vary very much from the unexpected normal range.

For example:

Animal	Plasma protein g/100 ml.	
	Range	Mean
Dog	6.1 - 7.8	6.72
Cow	7.4 - 10.2	8.30
Sheep		5.74
Horse		6.84

Specific Gravity

The simplest and most convenient method for the determination of specific gravity of urine is by use of the urinometer. The main limitation of its use is that it requires a large volume of urine, usually about 30 to 50 ml. The mixed non-aqueous solvent (benzene-chloroform) method for a drop of urine is well described by Coffin (loc. cit.) and requires no further comment except to state

XUM

that it is somewhat expensive in solvents as a large volume has to be used in order to measure the final specific gravity. The following method is based on the same principle except that it requires much less solvent for each estimation.

Principle.

A drop of urine is placed in a known volume of a mixture of non-aqueous solvents that has a specific gravity less than 1.000. The mixture is titrated with the heavier solvent until the urine drop just rises. The volume is noted and read off on a calibration curve.

Reagents and Equipment.

Chloroform and Benzene Mixture	In the proportions of 100 ml. chloroform and 500 ml. benzene. The solvents should be AnalaR or good laboratory grade. This quantity is sufficient for a calibration and 170 estimations.
Chloroform	As required.

Graduated measuring Cylinder, 50 ml.
Pipette, 5 to 10 ml. graduated in 0.1 ml.
Hypodermic Syringe, 1 ml.

Test Tubes, 3 in. x $\frac{3}{8}$ in.	Smaller tubes are not satisfactory as they will not hold the 3 ml. that are necessary for an estimation.

Urinometer.

Calibration of standard Solvent Mixture.

Place exactly 90 ml. of the solvent mixture in a graduated cylinder and also the urinometer. Now add chloroform very carefully until the urinometer reading is exactly 1.000. Note volume of chloroform added. Add exactly 3 ml. and note urinometer reading. Repeat with further additions of 3 ml., each time noting the specific gravity. It is unnecessary to continue above a value of 1.06. Divide the volume of chloroform added by 30 and plot the values obtained against the specific gravities obtained. This is necessary because 3 ml. of solvent mixture are used for each urine specific gravity determination. These determinations should be made as near 20° C. as possible or the temperature recommended on the urinometer, otherwise a correction will have to be made. The value of this is usually given by the makers of the urinometer. If the reagents used are AnalaR quality the specific gravity values will be guaranteed to within very close limits (about 0.5 per cent or less).

Procedure for Urine.

Measure exactly 3 ml. of the solvent mixture into a clean, dry tube. Add one drop of urine carefully so that it falls directly to the bottom of the tube. The drop size is *not* critical. Add chloroform by a clean, dry hypodermic syringe, shake tube carefully or invert if necessary. The end point is approached when the drop of urine begins to disintegrate into smaller droplets, and is reached when these remain stationary in the solvent. Note the volume and read off the specific gravity from the calibration curve.

XUM

Determination of the Approximate pH

The simplest method is by means of indicator paper. Litmus is not very satisfactory as it has a very limited range, and the tints near its neutral point are not very definite. A Wide Range, or Universal type paper such as manufactured by Messrs. B.D.H. is very satisfactory (*pH* 2 to 10.5).

Protein

With the exception of mucin in normal horse urine, the appearance of other proteins is usually pathological. The proteins are mainly albumins or globulins in varying proportions, according to the type and severity of the disease. Tests employed are those involving heat coagulation, and precipitation with salts of heavy metals, alkali salts, complex organic acids, alcohols, etc. Of this very wide range, sulphosalicylic acid is the most generally used. This is sensitive and can detect proteins in concentrations as low as 0.01 g. / 100 ml.

Reagents and Equipment.

Sulphosalicylic acid 5 per cent aqueous solution.
Test tubes, 2 in. x $\frac{3}{8}$ in. (or as available).
Black paper, preferably matte background.

Method.

One drop of urine is treated with one drop of sulphosalicylic acid in a test tube. The appearance of a turbidity or precipitate indicates the presence of proteins. Heavy discrete precipitates indicate a high concentration of protein of the order of 0.1 g. / 100 ml. or greater. Care should be taken to avoid excess of the sulphosalicylic acid as this may redissolve any precipitate. The method has been applied by King and Haslewood (1936) for the estimations of protein in human urine.

Reducing Substances

The most common reducing substances found in urine are glucose and lactose. The former usually occurs as the result of some disturbance to carbohydrate metabolism, the latter during lactation. Other substances such as glucuronides also occur in normal urines. The commonest tests involve the reduction of alkaline copper sulphate. The best known are Fehling's and Benedict's. Of the two, Benedict's is to be preferred since mixed Fehling's solutions A and B deteriorate on standing and may give a positive reaction on boiling without any sugar and in the presence of other non-specific substances. These include creatinine, uric acid, allantoin, homogentisic acid, nucleoprotein (probably due to the release of pentose) and glucuronides. The use of certain drugs, e.g., choral hydrate, antipyrine, and menthol, increase the normal output of glucuronides and may result in a definite "positive" with Fehling's or Benedict's. Glucuronic acid is itself a powerful reducing agent, but it does not appear to exist in the free state, as its main function is that of detoxicating agent. Several tests are described for the detection and estimation of the common sugars. Probably the most serviceable is the first described, namely, Qualitative Benedict's, although this requires the use of liquid reagent. The modification

using the solid reagent appears to be a little less sensitive. There are, however, commercially prepared tablets (Clinitest) that are substitutes for Benedict's solution.

1. *Benedict's Qualitative Reaction (solution)*

Reagents and Equipment.

Benedict's Qualitative Solution 500 ml. is enough for at least 250 tests.
Test tubes 3 in. x $\frac{3}{8}$ in. (or as available).

Method.

1 ml. of Benedict's solution and one drop of urine are boiled for one minute and allowed to cool. A coloured precipitate indicates the presence of a reducing substance.

A colour chart has been suggested by Nabarro (1954) and is similar to that of other workers. It is based on the normal "macro" procedure of Benedict's Test.

Colour	Approximate Sugar Concentration
Blue Blue-Green	nil to less than 0.1 per cent.
Clear Green	0.1 per cent.
Turbid Green	0.3 per cent.
Yellow Green	0.6 per cent.
Ochre	1.0 per cent.
Brick Red	2.0 per cent. or more.

2. *Benedict's Qualitative Reaction (solid)*

Reagents and Equipment.

Benedict's Mixture $CuSO_4.5H_2O$ 1.7 g., sodium citrate 17 g., anhydrous sodium carbonate 10 g. All well mixed and ground together. Keep in glass stoppered bottle. Sufficient for 750 to 1,000 tests.
Test tubes 2 in. x $\frac{3}{8}$ in.

Method.

Sufficient powder is placed in the tube to fill the concavity. Add one drop of urine and heat for about 10 to 20 seconds, or until a reaction commences. A cigarette lighter is quite adequate for this. If a reducing substance is present a colloidal precipitate is produced, the colour and quantity of which are approximately proportional to the concentration of reducing substance.

3. *Meyer's Modification of Benedict's Quantitative Reaction.*

Reagents and Equipment.

Benedict's Quantitative Solution 500 ml. is sufficient for 100 estimations.
Anhydrous Sodium Carbonate.
Hypodermic Syringe, 1 ml., or 1 ml. pipette.
Pipette, 5 to 10 ml. capacity, graduated in 0.1 ml.
Boiling Tube, 6 in. x 1 in.

Method.

5 ml. of Benedict's Quantitative Solution are mixed with about 5 ml. of distilled water and excess sodium carbonate in a boiling tube. The mixture is brought to the boil and while still boiling titrated with the urine. The end point is reached when there is complete disappearance of the blue colour. Note the volume of urine used.

Calculate the sugar present by the constants:

5 ml. of Benedict's Solution are reduced by 0.01 g. glucose *or* by 0.0132 g. lactose.

The titration is less accurate at the higher concentrations of sugar in urine (Fig. 1), and a small error in titration may result in a considerable final error.

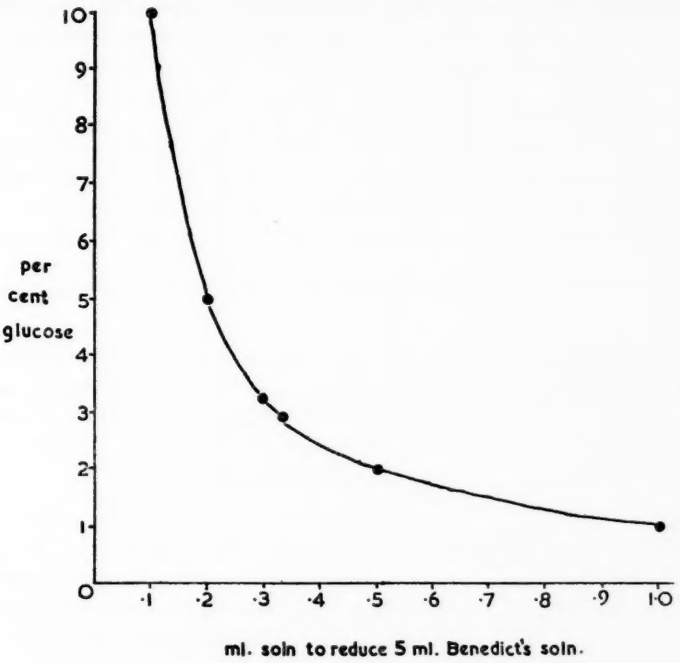

Fig. 1.
Relation between concentration of glucose in urine and quantity required to reduce 5 ml. of Benedict's Quantitative solution.

It may be necessary, therefore, to dilute the urine five or ten times in order to get a more accurate result. The author has employed the method using 1 ml. Benedict's solution, but the end point is more difficult to obtain accurately.

4. *Test on paper with p-anisidine.*

Principle.

This depends on the formation of a furfural type of compound from a sugar by the action of an acid, usually HCl. This is then coupled with an amine to give

XUM

a highly coloured complex. This reaction is widely used in the development of sugar spots in the paper chromatographic separation of sugars. This test, therefore, is the logical precursor of true paper chromatography.

Reagents and Equipment.

p-Anisidine in *n*-Butyl alcohol (Hough Jones and Wadman, 1950)	3 per cent, about 25 ml. Keep in glass stoppered dark bottle. Sufficient for 300 tests. Make freshly every two to three months.

Filter paper, Whatman No. 1.

Method.

Two drops of urine are placed about one inch apart on a filter paper. The paper is dried in air and one spot is treated with one drop of *p*-anisidine solution. After drying in the air for a few minutes the paper is heated in an oven at 100-110° C. for five to ten minutes or until a colour has developed. An alternate method of developing is to place the paper between two glass plates or between two Petri dishes and dry over a boiling-water bath. The presence of a sugar is demonstrated by the colouring of the spot. The shade and intensity of the colour should be noted and compared with the untreated spot. For the colours and reactions of specific reducing agents see under the next section.

5. *Chromatographic identification and separation of glucose and lactose.*

Principle.

Different sugars are separated from each other in partially water-miscible solvents in a vertical direction on filter paper. This is known as ascending chromatography. An excellent account of simple practical chromatography of the sugars is given by Varley (loc. cit.).

Reagents and Equipment.

Solvent Mixture (Hough Jones and Wadman, loc. cit.)	*n*-butyl alcohol 4 vols., ethyl alcohol 1.9 vols., water 1.1 vols.; about 200 to 300 ml. should be made up and kept in a glass stoppered bottle. This should be sufficient for many runs. Crude ethyl alcohol should *not* be used.

p-Anisidine in *n*-butyl alcohol 3 per cent (see previous section).
Whatman No. 1 Filter paper strips 14 in. x 1½ in.
Small quantities of glucose and lactose solutions, about 2 per cent strength.

"Kilner" or "Forster" Jar 2 lb. capacity.	The author has found these most satisfactory, although any jar of similar size with a good air-tight screw cap is satisfactory.

Method.

Put sufficient solvent in the jar so that the level is about half an inch from the convex bottom. Replace the lid firmly in order to establish a liquid-vapour equilibrium. Draw a faint pencil line across a Whatman No. 1 Filter paper strip about half to one inch from one end. By means of a small, tapered glass rod place

four small drops along this line, one for each of the two sugars and two for the urine. These spots should *not* exceed 2 to 3 mm. in diameter. Allow the paper to dry in the air for five to ten minutes. Adjust the length of the paper so that when it is finally lowered inside the jar the end will just dip below the surface of the liquid but still allow the pencil base line to be above the surface. Remove the lid of the jar and fix the other end of the strip to the inside of the lid by means of a small cardboard disc or else by a piece of "Sellotape" (Fig. 2). Lower

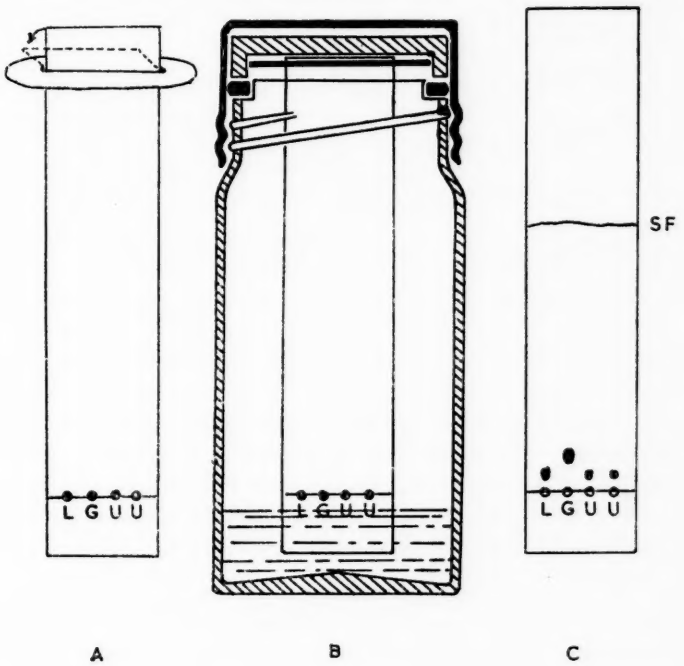

Fig. 2.
Ascending chromatography in a Kilner Jar. *A* is treated strip showing method of fixing into cardboard disc, *B* shows strip in position, *C* position of spots (approximate only) after developing, and solvent front.

carefully into jar, replace screw cap and tighten firmly. Place the jar in an atmosphere at an even temperature (not too cold) and protect from draughts by a corrugated cardboard cylinder.

After one to two hours, or longer, remove the strip, mark the distance the solvent has moved with a pencil and dry completely in the air or for a few minutes in the oven. *p*-Anisidine in butyl alcohol is then applied to the paper covering the base line and for a distance about two inches from it. While it is usual to apply a developing reagent by a fine spray it is simpler to "streak" it on very carefully and gently with a small camel-hair brush or even a glass rod. On no account should the developing agent be rubbed in. The treated paper is

XUM

heated in a manner similar to that described for the spot test in the previous paragraph. Note position and colour of the spots.

This list is, of course, very incomplete, and further information should be obtained from the literature.

Sugar	Properties of Spot
Pentoses	Move rapidly, develop a deep reddish purple colour very quickly. Appearance rare.
Glucose	Moves less rapidly than pentoses, yellow brown spot.
Fructose	Moves at similar but not identical rate to glucose. Bright yellow colour that develops better at temperatures a little higher than 100°C. (oven heat.)
Lactose	Moves very slowly. Yellow brown spot.
Glucuronic Acid	Free acid moves fairly rapidly, reddish spot. Glucuronides appear to move very slowly, give reddish pink spots, near base line.

The results of some typical runs of glucose and lactose obtained by the author at different time intervals are given at Fig. 3. It will be seen that even after one hour the separation of glucose and lactose is quite considerable and may be as much as 1 cm. Other constituents of urine have little or no effect on this chromatographic separation, although highly pigmented urines may leave a dark spot at the point of application.

The ultimate object of most chromatographic separations is to determine the R_f values of the substances under investigations. These values are characteristic for the substance in a specific solvent mixture under standard conditions. The definition is:

$$R_f = \frac{\text{Distance travelled by the substance}}{\text{Distance travelled by solvent front.}}$$

Under normal conditions R_f values are determined after 18- to 24-hour "runs." The present method, however, does not aim at very exact measurements but at the fairly rapid identification of one or two common reducing substances.

6. *Rapid fermentation tests for the differentiation of glucose from lactose and other reducing substances.*

Two useful test papers are now available for the detection of glucose. They are the TesTape (Eli Lilly & Co. Ltd.), and Clinistix (Ames Company, London, Ltd.). They are impregnated with a purified glucose oxidase, a vegetable peroxidase and *o*-tolidine. On contact with a glucose solution they change colour in a few seconds. This is due to the formation of hydrogen peroxide from the fermentation of the glucose, and the subsequent oxidation of the *o*-tolidine to a

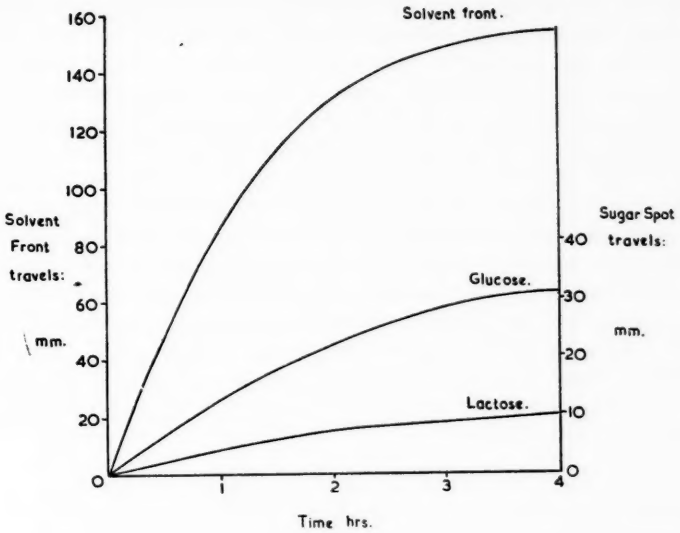

Fig. 3.

Ascending chromatography of glucose and lactose. Relation between distance travelled and length of "run."

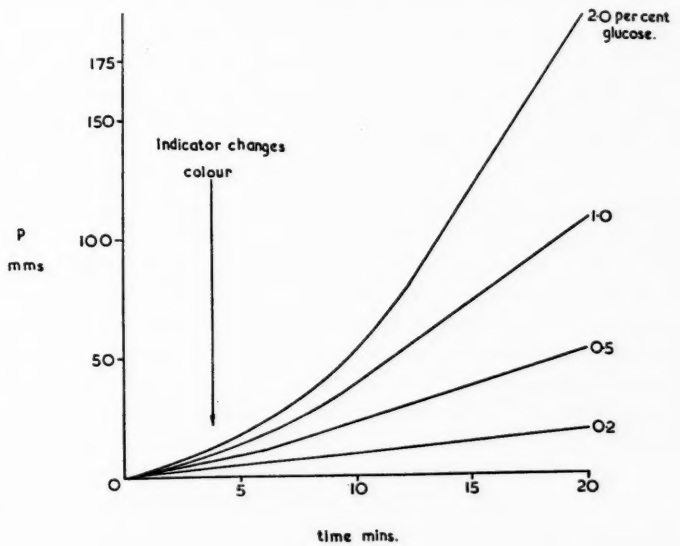

Fig. 4.

Fermentation of glucose by yeast at 37° C. 5 ml. of glucose urine solutions with strengths as shown. CO_2 measured by pressure difference (p) in mm. on a simple mercury manometer. Note that the colour change to the indicator often occurs before there is a very significant evolution of CO_2.

XUM

blue complex. Hunt, Gray and Thorogood (1956) and Tunbridge, Paley and Coulson (1956) have examined these test papers and conclude they are reasonably satisfactory for the qualitative detection of glucose. A limited number of investigations by the author on the urines of diabetic dogs supports this claim. The advantages of these papers lie in their complete simplicity, and the need for only a drop of urine. Taken in conjunction with Benedict's Qualitative Test they can be used to distinguish glucose from lactose and other reducing agents not fermented by glucose oxidase. A simple alternative to the test papers is based on the classical fermentation reaction of glucose by yeast. While most textbooks of practical biochemistry describe simple devices for the measurement of CO_2 produced during fermentation, few stress that the process commences rapidly and may be detected in a few minutes (*vide supra*) without measuring the CO_2. Thus other substances such as gluconic acid are produced and their contribution to the acidity may be detected by tests such as that described. The course of fermentation of glucose by yeast is shown in Fig. 4. Note initial lag in CO_2 production.

Reagents and Equipment for alternative yeast fermentation test.
Baker's Yeast.
Brom-cresol-purple (*pH* 5.2-6.8, 0.04 per cent solution; Messrs. B.D.H.)
Test tubes, 2 in. x $\frac{3}{8}$ in.

Method.

Place in a tube a small " bead " of yeast, 0.1 ml. of boiled, cooled and neutral urine, and one drop of the indicator solution. Shake well and warm at 37-40° C. If no waterbath is available, the tube may be kept in an inner pocket. If glucose is present the purple colour will be changed to yellow in a few minutes. If lactose is present there will be no colour change. At room temperature the colour change may occur in five to ten minutes.

Ketones

The most satisfactory method for the detection of ketones in urine (or in milk or blood) is by means of Rothera's Nitroprusside reaction. A well-known modification using solid reagents (Harrison, 1947; Varley, loc. cit.) is described below. It is sensitive to about 1 in 100,000. Gerhardt's test (10 per cent ferric chloride) may be used to identify acetoacetic acid (*not* acetone or β-hydroxybutyric) but it requires about 0.1 to 0.2 ml. urine.

Reagents and Equipment.

Nitroprusside Mixture	Finely-ground Sodium Nitroprusside 1 g., Ammonium Sulphate 20 g., Anhydrous Sodium Carbonate 20 g. The mixture is thoroughly mixed but *not* ground. It is kept in a well-stoppered bottle. This quantity is sufficient for about 1,000 tests.
Test tubes	2 in. x $\frac{3}{8}$ in.

Method.

A small quantity of the reagent is placed in a test tube to fill the concavity, or else placed on a filter paper. One drop of urine is added. The development

of a purple colour the intensity of which should be noted indicates the presence of ketones (acetone and acetoacetic acid but *not* β-hydroxybutyric acid).

Blood

A good standard chemical test for blood is the benzidine reaction. This is well known and is most sensitive. There are many variations of this and a good review of the relative merits of this and other tests is given by Caplan and Discombe (1951). Most modifications of the benzidine reaction have been aimed at producing solid reagents or tablets and to replace hydrogen peroxide solution. Thus Hyperol (urea/hydrogen peroxide) and Barium Peroxide have been used. Gregerson's modification (1926) has been adapted to a test-paper technique by Needham and Simpson (1952), but the paper appears to be too unstable for long storage.

Reagents and Equipment.

Benzidine Acetate 10 g.	A grade suitable for blood testing should be obtained.
Barium Peroxide 20 g.	The three substances are intimately mixed together and stored in a glass stoppered bottle. This is sufficient for about 1,000 tests.
Citric Acid A.R. 10 g.	
Glacial Acetic Acid A.R.	A few ml. in a small dropping bottle.
Test tubes	2 in. x $\frac{3}{8}$ in.

Method.

Sufficient of the reagent is placed in a test tube to fill the concavity, or else placed on a filter paper. One drop of urine is added. The appearance of a blue colour indicates the presence of hæmoglobin. The intensity of the colour is proportional to the concentration of the blood (hæmoglobin). The test is sensitive to about 1 in 500,000. If a colour does not appear although blood is suspected, it may be necessary to touch the spot with a small quantity of acetic acid. This usually brings up the colour.

Bile Pigments — Bilirubin, Biliverdin

A variety of tests for bile pigments have been described and are adequately reviewed and described in the literature. Lewis (1956) has recently revived the methylene blue test. This is very simple, especially when larger quantities of urine are available. The following modification of Fouchet's test is described by Coffin (loc. cit.) and is suitable for "drop" technique.

Reagents and Equipment.

Filter paper saturated with 10 per cent Barium Chloride (about 1 in. square).

Fouchet's Reagent. Ferric Chloride solution 10 per cent, 10 ml.
Trichloracetic acid 25 g.
Distilled water 100 ml.
This is sufficient for at least 2,000 tests.

XUM

Method.

One drop of urine is placed on the barium chloride paper and dried in the air. If pigments are present they will be adsorbed on the precipitated barium sulphate/phosphate and will be visible as a faint yellow-brown stain. Add one drop of Fouchet's reagent. If bile pigments are present a green or blue colour due to the formation of coloured oxidation products of bilirubin will be formed.

Acknowledgments

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OBSERVATIONS ON THE HÆMATOLOGY OF CALVES AND VARIOUS BREEDS OF ADULT DAIRY CATTLE

PART I (*Concluded*)

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Correlations of the Erythrocyte Picture.

The Mean Corpuscular Hæmoglobin Concentration. In *calves* this value ranged from 21 to 48 per cent (mean value 29.8 per cent) and showed little if any variation between the calves, irrespective of their age. The range for *adult* cattle was 23.7 to 54.6 per cent (mean value 33.7 per cent), although 81 per cent of the 588 recordings came within the limits of 30 to 40 per cent. It appears as if a figure below 23.7 per cent would serve as a guide to some abnormality.

The Mean Corpuscular Volume. The ratio between the erythrocyte count and the packed cell volume is an important one, and is expressed as the mean corpuscular volume (M.C.V.). A negative correlation exists in cattle between the erythrocyte count and the size of red cells, whereby low erythrocyte counts may have corpuscles above the average size and vice versa (Holman, 1952). This suggests that caution is necessary when interpreting the erythrocyte picture by a red cell count alone in the diagnosis of anæmia, and the observations of this series indicate that the age and breed of the animal must also be taken into consideration.

The M.C.V. of calves ranged between 28 and 112 c. micra with a mean value of 55.7, the main distribution coming within the limits of 40 to 60 c. micra. The extremes for adult cattle were 30.0 to 124.1 c. micra (mean 67.1), with a frequency distribution slightly higher than for calves, namely 50 to 80 c. micra. Differences will be found when comparing the results with those of authors in other countries and are accounted for by the type of anticoagulant used, which influences the packed cell volume. Mean corpuscular volume recordings of below 28 c. micra in calves, and 30 c. micra for adults suggests an inclination towards anæmia, and the necessity for further sampling.

The distribution curves of M.C.H.C. and M.C.V. indicate a tendency for these indices to skew to the left, which is in contrast to that described for the hæmoglobin and packed cell volume curves, which skew to the right.

The Diameter of Erythrocytes. An estimation of the diameter of erythrocytes is difficult because the number and size of red cells in cattle varies diurnally, and, according to Fiennes (1952), corpuscular diameter varies with the amount of water in the plasma. Using a micrometer eye-piece, little variation in the diameter of erythrocytes of calves during their first year of life was noted, the common range being 5.0 to 6.0 u, while for adults the diameter ranged between 6.6 and

XUM

7.9 u. Disagreement with other authors may be explained by the technique and the anticoagulant used.

Although no significant variations were found in the breeds studied, age seems to influence erythrocyte size, as in adults erythrocyte diameters were found to be larger during the age of 3 to 7 years.

The Morphology of Erythrocytes. In the blood films of calves, anisocystosis was displayed in the different age groups examined, particularly during the early weeks of life. Megalocytes appear to be a normal feature in cattle and were more numerous in the blood of calves than of adults. Only five normoblasts were found after an extensive search of all the smears made from calves up to the age of four weeks. No reticulocytes were observed, although Fraser (1930) found 0 to 1.4 per cent of reticulocytes in calves up to two days old.

Punctate basophilia was absent, although Fraser noted the occurrence of this in calves, and states " in some it is not present even within two hours of birth, and it has not been observed in the blood of healthy calves after 48 hours."

The erythrocyte picture of adults appears to present few characteristics of note. Many authors have reported that anisocytosis is not a prominent feature to be seen during the examination of normal cattle blood. Blount (1939), however, found anisocytosis to be a striking feature in healthy cattle of all ages, and these findings are in accordance with these observations. In contrast with the findings of Fraser, normoblasts were not seen in any of the blood films prepared from adult cattle.

Miscellaneous Measurements Connected with the Erythrocyte Picture.

Platelet Count. Owing to the difficulties associated with the technique of counting blood platelets, marked fluctuations in the recordings are to be expected; standard values should not be set up on the basis of their estimation, as these would vary according to the technique adopted. In calves the number of platelets varied between 150 and 526 x 10^3/cmm., and that of adults between 74 and 652 x 10^3/cmm., indicating that there is little or no difference between the counts of calves and adult cows. The platelets of calves, however, were found to be identified more easily than those of adults. Correlating the numbers of platelets with coagulation time indicated that in cattle, platelets are not indispensable for the coagulation of blood.

The Specific Gravity of Blood. Adopting the copper sulphate method described by Phillips and Van Slyke (1950), the specific gravity of calves' blood averaged 1052, and for adult cattle the mean value was 1049. The specific gravity figures for cows did not vary between the breeds, but Jersey cattle invariably had recordings at the lower limits of the range, and in accordance with their hæmoglobin values.

Although a correlation exists between the specific gravity and hæmoglobin content of blood, caution is necessary when making comparisons, as different techniques give different results.

The Fragility of Erythrocytes. Calves and adult cattle had a range of 0.30 to 0.50 per cent saline for erythrocyte fragility, with a mean value of 0.45 per cent, little variation occurring between the animals themselves, or the different breeds. Erythrocyte fragility did not appear to have any correlations with the other erythrocyte measurements.

The Leucocyte Picture.

The Total Leucocyte Count. The total leucocyte count of calves and adult cattle fluctuates considerably and a better estimation of the state of the leucocyte picture can be made by a consideration of a differential count.

Total leucocyte counts of between 4,500 and 15,000 per cmm. were made for calves, the main distribution being between 6,500 and 11,500 per cmm., (mean value $9.7 \pm 1.6 \times 10^3$). Such wide variations were found for adults that it would be illogical to give a mean value, but rather to consider the range that may be encountered for normal cattle. This range was 4,500 to 14,000 per cmm., the frequency of distribution being between 8,100 and 11,500 (mean value $9.1 \pm 1.4 \times 10^3$). Because of the fluctuations recorded, seasonal and breed differences were not in evidence, but a regression was noticed with advancing age.

No correlation could be determined between the total leucocyte counts and the other blood components, but Irwin and Bell (1938) suggested that a correlation existed between the total leucocyte and the erythrocyte counts, but this could not be established with these animals.

The Differential Count. The stress reaction already referred to causes characteristic changes in the leucocyte picture, not only in animals during fright and excitement but also at parturition and during œstrus. Similar changes occur in the calf at birth, due to pituitary-adrenal cortical activity. It is essential, therefore, that this factor be considered when interpreting a differential count, for in such circumstances varying degrees of neutrophilia/lymphopenia and eosinopenia are produced.

The Neutrophils. The neutrophils observed in the blood films were identified and classified according to Schilling's index, into those with immature and non-lobulated nuclei (Young and Band forms), and the mature or lobulated nuclear types (Adult forms). "Young" forms are scarce in the blood films of cattle, only 0.001 per cent being observed in the calf. In adults a percentage of 0.04 was recorded, which is higher than the figure usually given for this type of cell in cows. A percentage of 0.25 was recorded for one of the herds of the Guernsey breed, indicating that a "shift to the left" is possible even in healthy cows. "Band" forms were more frequently observed and represented 4 to 5 per cent of the total number of neutrophils seen in the calf, and 0.4 to 2 per cent in the adult animal. It seems that the percentages of the non-lobulated types of neutrophils vary from herd to herd, and may be a peculiarity to a cow, or a number of cows kept under a similar environment and management.

The percentage of adult neutrophils for calves varied within the range of 10 to 64 per cent (mean value 25 per cent), high values being recorded in the

XUM

early days of the calf's life. The range of 12 to 54 per cent obtained for adults (mean value 30 per cent) confirms the figures quoted by other workers. Breed differences ($p = <0.01$) were found on analysis of variance, and were associated mainly with Guernsey cows.

The Lymphocytes. These were classified as large and small, although Blount (1939) included a third type, namely medium-sized lymphocytes. Although the detailed morphology of the cells was not studied, controversial aspects of the subject were taken into consideration when examining the blood films. Only one Reider cell was observed, while vacuoles, although not numerous, were seen mainly in the lymphocytes of Ayrshire cattle.

The percentage of lymphocytes recorded for calves varied with age. During the first few days of life a percentage of 50 was obtained, which increased to 67 per cent by the end of the first week, a value which may be taken as the average for the next twelve months. Adult cattle had a lower mean value of 57 per cent with a range of 36 to 72 per cent. As with neutrophils, breed differences were found to exist on statistical analysis ($p = <0.01$), principally with the Guernsey cattle, differences which correspond to those noted in the recordings for neutrophils.

Eosinophils. In attempting to set standards for the normal range and mean values of the eosinophil count in cattle, it is essential to ensure that the animals are clinically healthy and free from parasitic infestations, and that at the time of bleeding there is no stress reaction which would result in an eosinopenia. Its absence has already been mentioned, and the negative correlation between the percentage of lymphocytes and the eosinophils supports this contention.

A characteristic feature of the blood picture of the calf at birth is the absence of eosinophils, which is attributed to the reaction of the pituitary-adrenal cortical system, due to stress at birth, or to the activity of this system in the dam which influences the calf via the placental circulation. During the first year of life the eosinophil count in calves varied according to age, increased values being recorded as the age of the animal advanced. Over the whole period a range of 0 to 15 per cent was obtained, with a marked increase in the numbers of eosinophils occurring during the age of 4 to 8 months, which could not be associated with a helminthiasis.

Eosinophils are more numerous in adult cattle, averaging 11.0 per cent, with extreme limits of 2.0 to 30 per cent. Insignificant variations were found to exist between the breeds, which was to be expected with such small percentages, but noticeable differences were noted between the cows within the herds ($p = <0.001$). Slight variations also occurred from month to month.

Monocytes. The percentage of this type of cell was found to fluctuate considerably in both calves and adults. In contrast to the observations of Blount (1939), vacuoles in this type of cell were not seen in any of the blood films examined, but agreement was reached with Fraser in that Karloff bodies do occur in cattle; Blount, however, stated that such bodies do not occur in cattle.

As with the other cellular components of the leucocyte picture, stress also affects the percentage of monocytes present by producing a monocytopenia.

Monocytes in calves ranged from 0 to 13 per cent (mean value 3.0 per cent), while the range of 0 to 8 per cent (mean value 2 per cent) recorded for adults is much lower than that given by Fraser (7 per cent). These differences, both for calves and adults, may be accounted for by the difficulty in distinguishing small monocytes from large lymphocytes. No breed variations were evident.

Diameter of Leucocytes. The diameter of the individual leucocytes varied considerably, and the more cells that were measured, the wider the range became. The neutrophils of calves varied in diameter between 9 and 14 u, which is in close agreement with that recorded for adults (6.6 to 13.2 u). Little variation was found in the neutrophil diameters of the various breeds, although Guernsey and Friesian cows had cells smaller than those observed in the other breeds.

In calves the small lymphocytes ranged from 7.0 to 10.0 u, and for adults 6.6 to 9.2 u, while the large lymphocytes of calves varied between 11.0 and 12.0 u, and 10.5 to 14.5 u in the case of adults.

While the eosinophils of calves varied in diameter between 8.0 and 15.0 u, it was noticed that those of adult cattle could be grouped as either large or small, a preponderance of one or the other occurring; the small cells having diameters ranging from 7.9 u to 9.2 u, while large eosinophils varied between 10.5 and 14.5 u. Exceptionally large eosinophils with diameters of 18.4 u may be observed occasionally.

Some difficulty is experienced in the identification of small monocytes and large lymphocytes, and this would account for the variations in diameter ascribed to the monocyte. Little variation was found in the diameters for this class of cell for calves and adults, 10.0 to 18.0 u being recorded in each case.

Correlations between the Cells of the Differential Count.

The relationship between the neutrophils and lymphocytes clearly indicates a negative correlation; such a correlation also occurs between the lymphocytes and eosinophils, which was to be expected when it was considered that stress had no influence on the results recorded. The differential counts of calves bled at birth, however, showed positive correlations in that reduced percentages for lymphocytes, eosinophils and monocytes were recorded, thus substantiating that stress influences the blood picture of a calf at birth. No other correlation between the different types of cells could be detected.

Summary

The hæmatological picture of 233 calves and 49 adult dairy cattle selected from the five main dairy breeds of this country have been studied over a period of two years.

The ages of the calves ranged from birth to 52 weeks and they were bled at weekly intervals; blood samples were taken from the adult cattle at monthly intervals.

XUM

The plan of investigation and the variations which occur have been outlined, and the normal ranges, mean values and frequency distributions of the constituents of bovine blood are recorded.

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(Part II will appear in the April issue.)

SOME OBSERVATIONS ON BOVINE PASTEURELLOSIS IN KENYA

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PART II

Immunology

(*a*) *Serological typing and identification of Past. boviseptica strains.*

It is necessary to determine whether serological types exist within the strains of *Past. boviseptica* used in vaccine production. The term "strain" that we have used arbitrarily to define cultures of the organism isolated from cases of bovine pasteurellosis is convenient but not scientific. If the existence of serological types within these strains can be proved, then the antigenic structure of each type must be determined as well as the immunising value of each type against homologous and heterologous types. In the absence of such precise information the preparation of a polyvalent vaccine, as at Kabete, from strains of *Past. boviseptica* selected at random, or through virtue of virulence alone, has scientific justification.

We first examined the possibility of a serological classification of the five vaccine strains on the basis of an agglutination test. As this test has been accepted by some workers and rejected by others as a means of type determination, a brief résumé of the literature is not out of place.

Cornelius (1929) noted that a number of strains of pasteurella of animal origin were feebly antigenic so that large amounts of culture had to be inoculated in rabbits, over a long period, to obtain agglutinating sera of reasonable titre; also, strains varied widely in "agglutinability," or became temporarily "inagglutinable," a difficulty overcome to some extent by using broth cultures instead of washed agar antigens. Formalinised antigens proved to be "inagglutinable." This worker finally adopted an agglutinin-absorption technique and classified 17 of 26 strains into four distinct serological groups. Brigham and Rettger (1935) prepared agglutinating sera from rabbits given increasing doses of formalinised culture at two-day intervals over several weeks and found the results of the agglutination test unsatisfactory; six strains of pasteurella were "inagglutinable" and several others practically so, and strains could not be typed by the test. Webster, *et al.* (1929) found that blue colony antigens of pasteurella of avian origin agglutinated strongly in antisera whereas fluorescent colony antigens did not. Anderson, *et al.* (1930), also working with an avian strain, noted that the antigenic structure of R and S types varied as did their agglutinability. Bhatnagar (1940) found that neither heat-treated nor formalinised cultures of *Past. pestis* produced satisfactory agglutinating sera in rabbits, but that, with improved antigens, an agglutination occurred, somatic and capsular, and that avirulent strains gave better titres than virulent; that boiled cultures quite devoid of capsular substance were necessary in raising pure somatic sera in rabbits and, most important, all anti-sera agglutinated with boiled

XUM

antigens. Priestley (1936) holds that pasteurella cultures are only agglutinable if all capsular antigen be removed by incubating cultures for three days at 37.5 C. In spite of the difficulties encountered by those workers, Rosenbusch and Merchant (1939) found no difficulty in obtaining high titre sera from rabbits injected first with heat-killed and later virulent cultures, and used simple washed agar cultures as antigen; thus it was possible to classify all strains examined in two main serological types, with a poorly defined third type. Tanaka (1926) found pasteurella cultures, whether inoculated in the killed or living state, are poor antigens. He could only obtain sera of low titre from rabbits and concluded that it is not possible to type strains by means of an agglutination technique. Both this worker and Roderick (1922) obtained clear-cut evidence of type

TABLE 2

Serum	Challenge Strain	TEST MICE		CONTROL MICE	
		TOTAL	NO. PROTECTED	TOTAL	NO. KILLED
No. 1	Type 1	4	2	2	2
"	Type 2	4	0	2	2
No. 2	Type 1	4	4	5	5
"	Type 2	4	4	5	5
No. 3	Type 2	5	1	5	5
"	Type 3	5	5	5	5
"	Type 4	5	5	5	0
No. 4	Type 2	5	5	5	5
"	Type 3	5	5	5	5
"	Type 4	5	5	5	0

differentiation by a complement-fixation test. Ysef (1935) came to the same conclusion, and evolved a precipitin test for determination of type specificity. Roberts (1947) examined 37 strains of pasteurella of animal origin by cross-protection experiments in mice. By using 21 strain specific sera prepared from rabbits it was possible to classify them into four distinct types by immunological differences supported by cultural and biochemical differences.

We now considered the possiblity of typing strains of Kenya origin after the technique of Roberts (1947) who kindly supplied his type 1-4 strains. These strains were examined at Kabete in comparison with the vaccine and certain field strains and proved identical on cultural and pathogenic standards as tested in mice. Broth cultures from each type were used in the preparation of type sera in rabbits according to Roberts' technique. It was found that rabbits at Kabete were prone to succumb to anaphylactic shock if increasing doses of antigen were given intravenously at four-day intervals, and injections were therefore spaced at 7 to 10-day intervals, this being the only deviation in technique. Rabbits were bled 10 days after the last injection and mouse protection tests carried out with the four type sera in adult mice averaging 18 gm. The test dose of each type culture, given intraperitoneally, was 0.1 ml. of a 10^{-8} dilution of buffered (*pH* 7.6) peptone water suspension of an 18-hour blood agar culture standardised at five opacity tube. Mice were given subcutaneously 0.2 ml. of the serum under test 24 hours before challenge. Table II gives the results of the tests.

Serum 1 protected against type 1 culture, but not against type 2. Serum 2 protected against type 2 culture but was not expected to protect against type 1 unless both cultures possess overlapping antigens. Serum 3 protected strongly against type 3 culture and not against type 2. Types 3 and 4 cultures apparently possess common antigens but this result was marred by an unexpected and unexplained fall in virulence of type 4 culture (which did not kill controls) to $LD_{50} = 10^{-2}$, as was assessed later. This method of typing strains appears, then, of undoubted value but further work on such lines was ruined by uncontrollable salmonella disease among the stock mice available for the work.

The sera from the mice used in the preceding experiment were examined for agglutinins. The antigens used in the agglutination test varied, viz.: (1) 18-hour and six-day broth cultures of blue colonies isolated on serum agar plates from type cultures 1 to 4 (Roberts) and N.C.T.C. 3195. (2) The same antigens boiled for 30 minutes. (3) The same treated with boiling alcohol then extracted with ether and suspended in normal saline. The density of the antigens was varied between opacity tubes 1 to 10. Antigens were made from types 1 to 4 strains at the third rabbit passage.

With none of these antigens did any trace of agglutination occur with type sera, either homologous or heterologous.

A series of rabbits were highly immunised severally against strains N.C.T.C. 3195 and 4308, Soy 1, Percival and Stanley, by means of heat-treated vaccines prepared from each strain. All rabbits were then given subcutaneously increasing doses of homologous virulent broth culture at 10-day intervals, viz., 0.1 ml., 0.25 ml., 0.5 ml. Rabbits were bled for serum 10 days after the last injection. The antigens used in the agglutination test were prepared from each strain in the same manner as in the previous experiment. None of the sera agglutinated such antigens.

Three steers which had been used recently in the experiment to compare the virulence of Soy 1, Percival and Stanley strains with N.C.T.C. strains 3195 and 4308 were given subcutaneously increasing does of virulent homologous culture at 10-day intervals and bled 10 days later. None of the sera gave a trace of agglutination against homologous antigens.

It is obvious from such experiments that the agglutination test is of no value in determining serological types within the species *Pasteurella boviseptica*.

General. The main object in this investigation has been to assay the prophylactic value of the formalinised bovine pasteurellosis vaccine in use in the Kenya field at the outset of the investigation in 1949. Formalinised vaccines have been used in countries other than Kenya and opinions on their efficacy vary. Jacotot (1926) found them of no value but Baloget (1926) considered that a useful immunity could be conferred on susceptible cattle: this appeared not to endure more than two months, but could be prolonged to a year or more by revaccination with virulent broth culture, a procedure usually free of risk,

XUM

though a few animals, especially among calves, might succumb later in a cachectic state. Didier (1929) used a formalinised vaccine against bovine pasteurellosis with apparent success in the Sudan, and Hall (1932) claimed good results with a similar vaccine in Nigeria, though immunity was short-lived. The vaccines used were 24 to 48-hour broth cultures of *Past. boviseptica* formalinised at 0.25 per cent.

Although the need of a reliable and proved vaccine is urgent in countries where bovine pasteurellosis is epizootic, very little sustained research seems to have been given to the problem of immunity. It is not proposed here to review the literature on the subject, but it can be said that no uniformity of opinion as to a really satisfactory method of protecting cattle against bovine pasteurellosis is indicated in the literature. In India, one of the worst affected countries, Holmes (1909) employed a method of sero-vaccination, the hyperimmune serum being obtained from buffaloes. Such an immune serum is still used in India to give a passive protection, but it is of doubtful value. The same worker also advised the use of an aggressin prepared from buffaloes, and claimed results as good as with sero-vaccination. Batelli (1944) considers an immune serum prepared by the hyper-immunisation of cattle of value both as prophylactic and curative. Doyle (1923), in India, utilised as a vaccine a strain of *Past. boviseptica* attenuated through the rabbit, and this vaccine is the first line of defence against bovine pasteurellosis in India. Baldrey (1907) and other workers have claimed an effective immunity following vaccination with both filtrates of *Past. boviseptica.* Priestley (1936) showed that a high grade immunity may be conferred on rabbits with cultures of *Past. boviseptica* heated at 56° C. for 30 minutes and insisted that high virulence and rich encapsulation are prime factors in selection of strains for vaccine production. Delpy and Restegard (1938) used a saponin vaccine with good results. Carter (1950) has compared the immunity levels in mice vaccinated with formalinised vaccine and a chick embryo vaccine, to the great advantage of the latter.

An attempt has been made in our investigation to explore the various techniques of immunity just mentioned as an alternative to the routine use of a formalinised vaccine. It is probably a fair observation that the problem of immunity in bovine pasteurellosis is still unsettled, and certainly no standard vaccine is available for use in those countries where such a vaccine is urgently needed. Little or no attention has yet been given in the veterinary field to the role of bacterial dissociation in the preparation of vaccines from strains of *Past. boviseptica.* Working with pasteurella of avian origin, Anderson, *et al.* (1930) showed that definite protection against subsequent infection with living S (virulent) forms of *Past. aviseptica* is afforded in pigeons by vaccines prepared from killed S type, killed R type, killed S-R type, and live R. type *Past. aviseptica,* in that ascending order. A mixture of both S and R types of organisms in a killed vaccine also appears to give a better immunity response than a vaccine prepared from either type alone. The virulence of the organism did not appear to be related to the immunising power of the vaccine. The antigenic structure of the S and R types was not identical. The vaccines used were blood agar

TABLE 3

Rabbit No.	How vaccinated	Immunity Test	Result
1	1.0 ml. heat treated	2.5 MLD Stanley St.	R. - 1
2	Percival vaccine, s/c	s/c, 15 days p.i.	R. - 1
C			D.^{X}-36-40hours
3	1.0 ml. heat treated	"	R. - 1
4	Soy 1 vaccine, s/c		R. - 1
C			D.^{X}-36-40hours
5	1.0 ml. heat treated	2.5 MLD Percival	R. - 1
6	Stanley vaccine, s/c	strain,s/c, 15 days p.i.	R. - 1
C			D.^{X}-36-40hours
7	1.0 ml. heat treated	"	R. - 1
8	Soy 1 vaccine, s/c		R. - 1
C			D.^{X}-36-40hours
9	1.0 ml. heat treated	2.5 MLD Soy strain	R. - 1
10	Percival vaccine, s/c	s/c, 15 days p.i.	R. - 1
C			D.^{X}-36-40hours
11	1.0 ml. heat treated	"	R. - 1
12	Stanley vaccine s/c		R. - 1
C			D.^{X}-36-40hours
13	1.0ml. heat treated	"	D.^{X}-36-40hours
14	"Vaccine" vaccine s/c		D. coccidiosis
C			D.^{X}-36-40hours
15	"	2.5 MLD Stanley	R. - 1
16		strain, s/c, 15 days p.i.	D.^{X}- 72 hours
C			D.^{X}-36-40hours
17	1.0 ml. heat treated	"	R. - 1
18	3195 vaccine, s/c		R. - 1
C			D.^{X}-36-40hours
19	1.0 ml. heat treated	"	R. - 1
20	4308 vaccine, s/c		R. - 1
C			D.^{X}-36-40hours
21	1.0 ml. heat treated	2.5 MLD 3195	R. - 1
22	Stanley vaccine, s/c	strain, s/c, 15 days p.i.	R. - 1
C			D.^{X}-36-40hours
23	1.0 ml. heat treated	2.5 MLD 4308	R. - 1
24	Stanley vaccine, s/c	strain, s/c, 15 days p.i.	R. - 1
C			D.^{X}-36-40hours

R. - = No reaction
1 = Immune
D.^{X} = Died, organisms recovered from heart blood and tissues
C. = Control

culture suspensions in normal saline killed at 56° C. for 30 minutes and carbolised at 0.5 per cent. This important work has support from other workers. Thus, de Kruif (1921, 1922) showed that rabbits could be very highly immunised against virulent S forms of *Past. lepiseptica* by avirulent R forms of the same organism, whereas a killed vaccine prepared from a virulent culture gave little protection. Hadley (1913), working with virulent and avirulent strains of *Past. aviseptica,* found that a high order of immunity could be obtained in rabbits vaccinated with avirulent cultures and later challenged with virulent.

(1) *The immunological relationship between strains of Past. boviseptica used in vaccine production at Kabete in 1949.*

The strains used were ".H S.," Percival, Soy 1, Stanley, Vaccine and Soy 2. The last strain had been discarded in routine vaccine production at the commencement of the investigation as it seemed undesirable to include a strain of ovine origin in a vaccine intended solely for cattle. " H.S." strain was rejected at the same time because of loss of virulence. These two strains were replaced in routine vaccine preparation by N.C.T.C. strains 3195 and 4308.

Rabbits were immunised by heat-treated vaccines (see later) prepared from each vaccine strain and challenged in batches with heterologous strains. Table III gives the results of the cross-immunity tests. The immunological identity of strains Percival, Stanley, Soy 1, N.C.T.C. 3195 and 4308 is at once apparent. Rabbits vaccinated with " Vaccine " strain vaccine showed little evidence of immunity and this strain was, therefore, considered of low antigenic value; it has already been noted that it was practically avirulent for rabbits. " Vaccine " strain was, therefore, discontinued from further use in vaccine production. The 19 field strains isolated from outbreaks of bovine pasteurellosis in Kenya, and previously referred to, were shown by the technique to be immunologically identical with strains Soy 1, Percival, Stanley, N.C.T.C. 4308 and 3195 which were now alone used in the preparation of vaccine issued from Kabete.

(2) *The immunity induced in cattle by subcutaneous inoculation of strains of Past. boviseptica of low virulence.*

One steer was given subcutaneously 1.0 ml. 18-hour broth culture of strain Soy 1. It evinced considerable œdema at the site of inoculation, with fever, persisting for seven days. This animal was challenged 20 days later with 2.0 ml. 18-hour broth culture of the same strain and showed neither local reaction nor fever. This steer was again challenged one month later with 2.0 ml. 18-hour broth culture N.C.T.C. 3195 strain; it showed slight local reaction for 48 hours and no fever, whereas two control steers died of acute pasteurellosis within 48 hours. A second steer was inoculated subcutaneously with 2.0 ml. 18-hour broth culture Stanley strain and showed moderate and localised œdema with mild fever for three days. This animal was challenged one month later with 2.0 ml. 18-hour broth culture of the homologous strain, without reaction. Thirty days later it resisted challenge with 2.0 ml. 18-hour culture Soy 1 strain. Challenge was made by the subcutaneous route and all cultures used were proved

of standard virulence in rabbits. It would appear, then, that recovery from the experimental disease evoked by the subcutaneous inoculation of living culture of low virulence results in a solid immunity. It would also indicate that cattle could be immunised effectively by living broth cultures of *Past. boviseptica* of low virulence provided that the degree of attenuation of such cultures could be "fixed." These results also indicate, as already shown on the basis of cross-immunity tests in rabbits, that strains Stanley, Soy 1 and N.C.T.C. 3195 are immunologically identical.

(3) *The biological assay of Kabete bovine pasteurellosis vaccine.*

(*a*) *In mice and rabbits.* The polyvalent vaccine in use in 1949, at the outset of the investigation, was prepared from strains Soy 1, Stanley, Percival, "H.S.," "Vaccine" and Soy 2. These vaccine strains were maintained in blood agar

TABLE 4

No. of Animals	Vaccine	Dose	No. of Animals Challenged NCTC 3195	Challenge 30 days after vac. Alive	Dead	Percentage Protected
20 mice	Percival	0.05 ml s/c	20	7	13	35
"	"	0.1 ml. s/c	18	11	7	62.2
"	Soy	0.05 ml s/c	20	4	16	20
"	"	0.1 ml. s/c	20	6	14	30
"	Stanley	0.05 ml s/c	18	5	13	27.8
"	"	0.1 ml. s/c	19	8	11	42
"	Controls	-	20	-	20	Nil
1 rabbit)	Percival	0.01 ml s/c	1)			
1 rabbit)	"	0.1 ml. s/c	1)	0	3	"
1 rabbit)	"	1.0 ml. s/c	1)			
1 rabbit)	Soy	0.01 ml s/c	1)			
1 rabbit)	"	0.1 ml. s/c	1)	0	3	"
1 rabbit)	"	1.0 ml. s/c	1)			
1 rabbit)	Stanley	0.01 ml.s/c	1)			
1 rabbit)	"	0.1 ml. s/c	1)	0	3	"
1 rabbit)	"	1.0 ml. s/c	1)			
3 rabbits	Controls	-	3	0	3	"

slants and passaged in rabbits at three-monthly intervals. Ten litre flasks filled with ordinary beef broth *pH* 7.4 were seeded with fresh broth cultures of the six vaccine strains and incubated for 24 hours at 37.5° C., then formalin was added to give a one per cent concentration. The density of this vaccine was approximately one-tenth opacity tube 1. The dose of the vaccine for cattle was 1.0 ml. given subcutaneously and immunity was said to last for three to six months. Preliminary work with these vaccine strains had, as previously stated, shown strains "H.S.," "Vaccine" and Soy 2 to be unsuitable for further use in routine vaccine production, and further supplies of the vaccine were, therefore, prepared from strains Soy 1, Percival and Stanley alone. This step was justified by the immunological identity of these three strains with field strains isolated early in the investigation. Indeed, with this observation there seemed little justification for preparing a vaccine from three strains which were immunologically identical and probably of equal valency; if this view be

XUM

accepted the vaccine comprising these strains was in no sense polyvalent. Whether there did exist a variation in immunologic values between these three strains was now examined and, at the same time, the measure of immunity that could be expected in laboratory animals vaccinated against each of the strains in turn. Accordingly, the standard type vaccine was prepared from each strain. Table IV gives the results of the test of these vaccines in mice and rabblits. Rabbits were challenged with one M.L.D. strain N.C.T.C. 3195 and mice with 0.1 ml. 10 LD_{50} (mouse) of the same strain.

Certain general conclusions may be inferred from the present experiment. First, a vaccine prepared by the method described has feeble immunising capacity against a strain of similar immunological type, albeit a strain of greater virulence; thus, a measure of protection in mice better than a mean of 27.6 per cent at the lower and 44.7 per cent at the higher dose rate was expected. The latter figure would seem to indicate that a higher immunity response follows an increased dose of vaccine. It is obvious that the vaccine at the dose given has no protective value whatever in rabbits given a moderate challenge. In this connection, a direct slide examination of the vaccine prepared by the standard method showed poor capsule content, a fact with which we were at once impressed, since it is generally agreed by other workers that it is the capsular substance of the organism that is concerned in the mechanism of immunity. It will be noted that when rabbits were given a dose of the vaccine equal to that ordinarily given to cattle no immunity resulted. Second, it seems reasonable to consider, from the results of the experiment, that the three strains used are approximately equal in immunising property.

(*b*) *In cattle.* Ten healthy young steers were each given 1.0 ml. standard vaccine. Six were challenged 15 days later with 2.0 ml. 18-hour broth culture strain N.C.T.C. 3195 given subcutaneously, along with one control. One vaccinated steer survived and the remainder died as rapidly as the control. Four were challenged one month after vaccination with 1.0 ml. 18-hour broth culture strain 3195, and again one steer survived challenge. The challenge dose may be considered somewhat severe but it was selected since cattle immunised by living culture of low virulence easily withstood it; and further, we have deemed that if a killed vaccine is of practical prophylactic value it should give as potent an immunity as that conferred by the subcutaneous inoculation of live culture of low virulence. This was, at least, the standard laid down in this investigation. It is obvious from the preceding experiments that, on this standard, the existing vaccine fails quite conclusively, and it is very doubtful whether the vaccine is of any prophylactic value in the field, an observation that is not unwarranted from well-substantiated records of failure to control outbreaks of bovine pasteurellosis.

(4) *The immunising effect of massive doses of standard vaccine in laboratory animals and cattle.*

The fact that mice vaccinated with large doses of the vaccine showed some measure of immunity on subsequent challenge with virulent culture, a

measure of immunity apparently enhanced by an increased dose of vaccine, indicates that, however disappointing the results in cattle and rabbits, the vaccine is not entirely devoid of antigenic value. It has been noted that the vaccine is poor in capsular substance. If cattle were given massive doses of the vaccine would the increased supply of capsular material made available to the tissues result in a reasonable immunity? This proposition was examined in rabbits. Ten young rabblits were each given 2.0 ml. of the standard vaccine and challenged three months later with one M.L.D. of Soy. 1, Percival and Stanley strain, one strain to each batch challenged. The immunity was solid, five controls succumbing to acute pasteurellosis within 24 to 48 hours.

Similarly, six young susceptible steers were vaccinated with 50 ml. vaccine, a dose that was tolerated well. Three were challenged with 1.0 ml. 18-hour broth culture strain N.C.T.C. 3195 15 days later and three one month after vaccination, along with two controls. Neither local œdema nor fever was shown in the vaccinated cattle whereas this was maximal in controls which died within three days from acute pasteurellosis. This experiment was repeated on 24 susceptible cattle. At one month after vaccination, seven of eight were solidly immune to challenge with 2.0 ml. 3195 culture which killed two controls. The same result was seen in eight given the same challenge at three months after vaccination. At six months after vaccination, six cattle vaccinated were proved solidly immune. Thus, we have definite proof of the proposition that the *standard vaccine will give a reasonable immunity in cattle provided sufficient antigen is given.* Also, cattle so immunised can withstand the exacting challenge tolerated by cattle vaccinated with living cultures of low virulence.

(5) *The preparation of a new, concentrated bovine pasteurellosis vaccine.*

It follows from the previous experiments that the production of a vaccine of high immunising potency was now within reach. It was not evident that it is the amount of antigen, probably capsular substance, as determined by the concentration of organisms rich in this material, that is a factor of major importance in the production of a formalised vaccine of high immunising value.

Such a vaccine was prepared as follows:—

Strains Stanley, Percival and Soy 1 were retained as vaccine strains and two strains of high virulence, viz. N.C.T.C. 3195 and 4308 added. Eighteen-hour serum broth cultures of each strain were seeded on semi-soft serum agar plates and after 18 hours incubation at 37.5° C. typical blue colonies of each strain were fished and sown in serum broth tubes. Twelve-hour cultures of the latter were seeded on a hæmolysed blood agar medium prepared as follows: Bovine blood collected aseptically was hæmolysed with 10 volumes sterile distilled water, sterilised by filtration, and 0.1 ml. of the filtrate added to 100 ml. molten nutrient agar *pH* 7.4 in Roux flasks which were then shaken to disperse the medium over a wide surface for growth. After seeding, the flasks were incubated for 18 hours at 37.5° C. and then the growth in each flask was emulsified with 0.25 per cent formalin to match the opacity tube 10. Suspensions were examined for satisfactory capsule production and then pooled and stored

XUM

for three days at incubator temperature. Sterility and safety tests of the vaccine were done in the usual manner.

Biological assay of new vaccine: Comparion with standard vaccine.

(*a*) *In mice.* Fifty mice were vaccinated with 0.25 ml. standard vaccine and 50 with the same dose of the new vaccine. Challenge was made, 30 days after vaccination, with 0.1 ml. 10 LD_{50} (mouse) strain N.C.T.C. 3195. Table V gives the results.

(*b*) *In rabbits.* Ten rabbits were vaccinated, each with 0.5 ml. new vaccine, and 10 with 2.0 ml. standard vaccine. Both batches were challenged one month

TABLE 5

MICE	HOW VACCINATED	NUMBER CHALLENGED	ALIVE	DEAD	PERCENTATE PROTECTION
50	0.25 ml. standard vaccine, s/c	46	17	29	57
10	Controls	10	-	10	
50	0.25 ml. batch 48 new vaccine s/c	10	10	-	100
2	Controls	2	-	2	
10	0.25 ml. batch 49 new vaccine s/c	10	10	-	100
2	Controls	2	-	2	
10	0.25 ml. batch 58 new vaccine s/c	19	9	-	100
10	Controls	10	-	10	
10	0.25 ml. batch 60 new vaccine s/c	10	10	-	100
10	Controls	10	-	10	
10	0.25 ml. batch 76 new vaccine s/c	8	8	-	100
5	Controls	5	-	5	
10	0.25 ml. batch 76 new vaccine s/c	8	8	-	100
10	Controls	10	-	10	

after vaccination with 10 M.L.D. strain N.C.T.C. 3195, along with five controls. All rabbits given standard vaccine succumbed as rapidly as controls; all rabbits given new vaccine withstood challenge, without evincing any symptoms of illness.

(*c*) *In cattle.* Table VI gives the results of challenge of cattle vaccinated either with standard or new vaccine, at varying intervals of time and at various dose rates. The challenge dose was fixed at 2.0 ml. 18-hour broth culture strain N.C.T.C. 3195. These results indicate that a flat dose of 5.0 ml. of the new vaccine will immunise for a period of not less than one year. An equivalent

dose of the standard vaccine conferred no immunity on cattle three months after vaccination. The same effect is seen using smaller doses of both vaccines; thus, 2.5 ml. new vaccine protected strongly at six months, but 2.0 ml. standard vaccine did not protect at one month. A dose of 2.0 ml. of new vaccine seems to be about the threshold as half this dose did not protect cattle at one month.

TABLE 6

No. of Cattle	How Vaccinated	Number Challenged	Alive	Dead	Percentage Protection
Challenged at one month					
8	5.0 ml. new vaccine, s/c	8	8	-	100
6	1.0 ml. new vaccine, s/c	6	-	6	nil
3	2.0 ml. standard vaccine, s/c	3	-	3	nil
2	Controls	2	-	2	
Challenged at three months					
7	5.0 ml. new vaccine, s/c	7	7	-	100
5	5.0 ml. standard vaccine, s/c	5	-	5	nil
2	Controls	2	-	2	
Challenged at six months					
8	5.0 ml. new vaccine, s/c	8	8	-	100
11	2.5 ml. new vaccine, s/c	11	11	-	100
3	Controls	3	-	3	
Challenged at one year					
5	5.0 ml. new vaccine, s/c	3	3	-	100
2	Controls	2	-	2	

The evidence is that the new vaccine is a very highly potent immunising agent at an economic dose rate of 2.5 ml. in cattle; in comparison, the same dose of the standard vaccine has no effect in evoking a useful immunity.

At this stage it became evident that a high degree of immunity could be obtained by the use of a vaccine containing a relatively large quantity of antigen. Such a vaccine prepared by the method already described, or by some

similar concentration method, would be highly efficient, but would be expensive to produce. In the hope, therefore, of finding a cheaper method of at least equal efficiency in protecting cattle against bovine pasteurellosis, the following methods were investigated: (*a*) chick embryo vaccine described by Carter (1950), (*b*) aggressins, (*c*) immune serum, (*d*) avirulent R. culture, (*e*) broth filtrate, (*f*) dead culture, (*g*) heat-treated vaccine, (*h*) saponin vaccine.

TABLE 7

Mice

DILUTION OF CHALLENGE	ALIVE	DEAD	TOTALS Alive	Dead	PERCENTAGE MORTALITY
A. New vaccine group					
10^{-4}	0	4	0	7	100
10^{-5}	3	1	3	3	50
10^{-6}	2	2	5	2	28.5
10^{-7}	4	0	9	0	0
10^{-8}	4	0	13	0	0
B. Carter's chick embryo vaccine group					
10^{-4}	0	4	0	18	100
10^{-5}	0	4	0	14	100
10^{-6}	0	4	0	10	100
10^{-7}	1	3	1	6	85.7
10^{-8}	1	3	2	3	60
C. Controls					
10^{-4}	0	4	0	19	100
10^{-5}	0	4	0	15	100
10^{-6}	0	4	0	11	100
10^{-7}	1	3	1	7	87.5
10^{-8}	0	4	1	4	80

(*a*) Carter's work claims that a chick embryo vaccine gives a much higher order of immunity than formalin-killed culture vaccines. We have compared the immunological values of the new formalinised vaccine with chick embryo vaccine prepared from strain N.C.T.C. 3195 according to Carter's formula. Adult white mice averaging 18 gm. were used. The first batch was given 0.25 ml. new vaccine intraperitoneally and the second the same dose of chick embryo vaccine by the same route. Mice of both batches were revaccinated 48 days after the first vaccination by the same method and challenged 23 days later. The challenge dose was 0.1 ml. intraperitoneally of serial tenfold dilutions of allantoic fluid freshly procured from third egg passage of strain N.C.T.C. 3195. Four mice of each batch were challenged at each dilution, along with equal controls, and percentage mortalities four days after challenge calculated. All deaths were proved to be due to pasteurella septicæmia.

The results are calculated on the Muench-Reed formula and although LD_{50} in controls extended beyond 10^{-8} dilution the results shown in Table VII are sufficiently clear cut. Thus, at the challenge titre 10^{-7}, 100 per cent survival was seen in mice vaccinated with the new vaccine in comparison with 100 per cent mortality in mice vaccinated with the chick embryo vaccine.

The chick embryo vaccine had little prophylactic value in comparison with the new vaccine. The experiment was repeated with similar results. It was

observed, incidentally, that chick embryo passaged 3195 strain was feebly capsulated in comparison with blood broth culture of the same organism. It was decided on the basis of these experiments that the chick embryo vaccine could not be accepted as an alternative for the new formalinised vaccine.

(*b*) *Aggressins*: This product was prepared from steers used as controls in immunity experiments with the new vaccine. The amount of œdema fluid that can be collected from the local lesions following the subcutaneous inoculation of virulent culture is very variable and it was exceptional to find a massive spreading œdema that would yield a large quantity of aggressin. The concentration of organisms in such material was variable, sometimes teeming and often very scanty. Aggressin used in experiments was selected for high organism content.

Five cattle were inoculated subcutaneously, each with 5 ml. freshly prepared strain N.C.T.C. 3195 aggressin formalinised at 0.5 per cent and stored at —5° C. for three days, which effected complete sterility. A slight transient swelling followed inoculation. The cattle were challenged 22 days later with 1 ml. 18-hour broth culture strain N.C.T.C. 3195. Three cattle died of acute pasteurellosis two to four days after challenge. Œdema fluid obtained from a steer inoculated subcutaneously with strain N.C.T.C. 3195 was filtered through a Sietz E.K. disc, which effected sterility. Seven cattle were each given 5 ml. subcutaneously with a negligible reaction and challenged 22 days later as in the previous experiment. Two died of acute pasteurellosis three days after challenge, four were solidly immune and one steer was stolen during experiment. Six steers were subcutaneously inoculated with filtered aggressin prepared from strain N.C.T.C. 4308, four steers with 20 ml. and two with 5 ml., the larger dose being well tolerated. All were challenged 34 days later with 1 ml. strain N.C.T.C. 4308. Two cattle given the higher dose and one the lower died after six, two and seven days respectively, pasteurellosis again being confirmed. There was thus little evidence that aggressin would serve as a useful substitute for the new formalinised vaccine.

(*c*) *Serum prophylaxis*: A steer which had recovered from a severe reaction to the subcutaneous inoculation of 2 ml. 18-hour broth culture Soy 1 strain was given 5 ml. subcutaneously one month later, and after ten days 10 ml. of the same strain, and bled for serum one month later. Two rabbits were each inoculated subcutaneously with 1 ml. of this serum and simultaneously on the opposite side of the neck with 1 ml. 18-hour broth culture strain Soy 1. Neither rabbit reacted and two controls succumbed to an inoculation of one M.L.D. of the same culture. The same dose of serum protected two rabbits against one M.L.D. of N.C.T.C. 3195 strain which killed two control rabbits; Stanley strain killed serum-protected as well as control rabbits. The serum was also tested in cattle. Of two steers inoculated subcutaneously with 5 ml. serum and simultaneously given 5 ml. 18-hour broth culture Soy 1 strain on the opposite side of the body, one did not react and the other reacted very severely, with recovery in five days. Of two steers similarly tested with 10 ml. serum, one gave a mild reaction and the second died from acute pasteurellosis. One steer tested in the same way with 15 ml. serum did not react, whereas a steer given 20 ml. serum survived after a very serious illness. Of three control steers, two given 1 ml. culture

subcutaneously died of acute pasteurellosis, and one given 5 ml. was critically ill and recovered. Two steers given 20 ml. serum and tested in the same way with 1 ml. strain N.C.T.C. 4308 died within three days, as did two steers given the same dose of serum and strain N.C.T.C. 3195.

One steer was given subcutaneously 50 ml. standard vaccine and one month later challenged with 1 ml. fully virulent broth culture strain N.C.T.C. 3195. A bad reaction with recovery followed. This animal was again inoculated thrice with 5 ml. virulent culture of the same strain at monthly intervals without reaction and bled for serum one month after the last inoculation. Three rabbits subcutaneously inoculated with one M.L.D. of N.C.T.C. 3195 and given 1 ml. of the bovine serum on the opposite side of the body died as quickly as controls.

No conclusion can be drawn from these experiments. It is conceivable, perhaps probable, that an immune serum has *some* prophylactic value in countering an acute experimental infection.

(d) *Avirulent strains as immunising agents*: Mention has been made of a culture of strain R.M.X. which developed rough characters in subcultures made from original broth cultures stored for 90 days at room temperature and which became avirulent in rabbits at a standard test dose, i.e., one M.L.D. This culture gave a granular deposit in broth with clear supernatant, but S colonies developed in small numbers on agar plates. Broth cultures were made from the spreading margin of R colonies, incubated for 12 days at 40° C., then plated on 3 per cent agar. Plates were incubated under the same conditions as broth cultures. The process was repeated until only true rough colonies developed on agar plates. Two rabbits were given subcutaneously 0.25 ml. broth culture; both showed mild fever and transient swelling at the site of inoculation and 20 days later were solidly immune to challenge with one M.L.D. strain N.C.T.C. 3195 which killed controls. The broth culture used in this experiment was serially subcultured in broth under the foregoing conditions and at the 10th subculture rabbits were inoculated subcutaneously with 0.25 ml., a mild reaction ensuing, and were proved solidly immune 20 days later. This experiment was repeated in six rabbits each given 0.5 ml., and four steers given 1 ml. culture. The latter showed fever up to 105° F. lasting for three days with moderate local swelling. The rabbits resisted challenge 20 days later with one M.L.D. strain N.C.T.C. as did the steers given 1 ml. 18-hour broth culture of the strain, this dose killing one control steer in 48 hours. Eight further subcultures of broth culture used in this experiment were made, again under the conditions stated above. The 8th subculture yielded only R colonies and two rabbits withstood 0.5 ml. subcutaneously without reaction. This culture was then subcultured on serum agar plates, incubated four days at 37.5° C. and still gave apparent R colonies, one of which was subcultured in serum broth incubated in the same manner. Two rabbits were inoculated subcutaneously with 0.25 ml. of this culture and both reacted badly; one died nine days after inoculation with lesions of septic peritonitis and myocarditis, and purulent pneumonia with extensive septic cellulitis extending from the site of inoculation; the organism inoculated teemed in the pus but was not recovered in cultures made from the heart blood. Cultures on serum agar plates from the

XUM

purulent lesions yielded S colonies entirely. It was, therefore, quite obvious that the attempt to develop a pure R variant of strain RMX had failed in this experiment. The broth culture used in the experiment was now subcultured ten times in sulphapyridine broth and plated on serum agar. R colonies alone developed. Subsequent subcultures on serum agar plates were made from the spreading margin of R colonies and cultures. Two calves were now given 0.25 ml. of this culture which caused mild reaction, and both were challenged one month later with 1 ml. strain N.C.T.C. 3195 which killed the one control steer. Five cattle were inoculated subcutaneously with 1 ml. broth culture, none reacted and all were solidly immune 20 days later to the same challenge. The culture was subcultured in broth for five months and tested again in cattle. Three steers were given 0.5 ml. culture and reacted slightly. No reaction was seen in four rabbits given 0.25 ml. But four rabbits collapsed in six to eight days when given 1 ml. culture, and of six steers given 5 ml. subcutaneously, three gave bad reactions with death in six to ten days. Smooth type colonies only were isolated from the tissues of both rabbits and steers. It was now obvious that failure had attended this further effort to procure a true R variant of strain RMX. But the indication was that such would be, if capable of attainment, of the greatest value as an immunising agent in the control of bovine pasteurellosis. Also, if cattle could be immunised with a fractional dose of such a product effectively, the saving in the cost of production of the present new vaccine would be considerable; for example, one 10-litre flask of broth would yield, say, 40,000 cattle doses as against 400 doses of the new vaccine. Further work remains to be done on this aspect of immunity.

(*e*) *Broth filtrate as immunising agent*: Recently rabbit passaged strains Soy 1 and N.C.T.C. strain 4308 were grown 24 hours in serum broth incubated at 37.5° C. Each culture was filtered through Seitz E.K. discs. Two rabbits were inoculated subcutaneously each with 1 ml. Soy 1 filtrate and two with N.C.T.C. strain 4308 filtrate. The purpose was to determine whether a toxin is developed in broth cultures. No rabbit exhibited either local swelling or fever. The test was repeated with filtrates of 5- and 10-day cultures of the same strains, with like results. All rabbits were challenged 20 days after inoculation with one M.L.D. homologous strain and died as quickly as controls.

(*f*) *Dead cultures as immunising agent*: It has been shown already that a normal saline wash of N.C.T.C. strain 3195 incubated 24 hours on blood agar and stored at laboratory temperature is fully viable at 46 days but dead ten days later. Rabbits and mice inoculated with this dead antigen acquired no immunity.

(*g*) *Heat-treated vaccine as immunising agent*: The solid immunity conferred on rabbits by a heat-treated vaccine (Table III) could be conferred equally on cattle. Six young steers were inoculated subcutaneously with 5 ml. vaccine prepared from strain N.C.T.C. 3195, in the same way as vaccine was prepared for rabbits. All were challenged subcutaneously six weeks later with 1 ml. 18-hour broth culture strain N.C.T.C. 3195. Two reacted slightly with local swelling and some fever, while one control steer and four control rabbits, the latter given one M.L.D. culture, died. Five steers given 1 ml. of the vaccine and challenged in the same way were equally protected. It was evident that this

vaccine has a higher immunising value than the new formalinised vaccine which definitely failed to protect at a dose rate of 1 ml. It was considered that without the addition of a suitable preservative which might alter the antigenic quality such a vaccine might prove impracticable in the field, but further research on the problem is required.

(*h*) *The saponin vaccine* described by Delpy and Restegard is commended by Curasson. This is a soluble vaccine prepared by diluting a suspension of *Past. boviseptica* grown on agar with 10 parts of a 5 per cent solution of saponin in distilled water. At the end of 24 hours the suspension is clear and sterile. The cattle dose is 1 to 3 ml. Cattle can tolerate, 12 days later, 50 lethal doses challenge strain. The vaccine was prepared to formula from strain N.C.T.C. 3195. Six rabbits inoculated subcutaneously with 0.25 ml. 1/10 saline dilution of the vaccine maintained for two days at —5° C. died of acute pasteurellosis. The vaccine was safe at four days and six rabbits withstood 0.25 ml. Five steers given 2 ml. four-days-old vaccine subcutaneously reacted severely and were challenged one month later with 0.25 ml. virulent broth culture strain N.C.T.C. 3195. All died three to four days after challenge. The same culture at the same dose was used in the challenge of five steers given 20 days previously 2.5 ml. new vaccine; all were proved solidly immune. The six rabbits given 4-day stored saponin vaccine succumbed to challenge 20 days later, with one M.L.D. strain N.C.T.C. 3195.

It is evident that saponin vaccine as prepared could not be recommended as an alternative for the new formalinised vaccine.

Production of the new formalinised vaccine.

An examination of the various methods of protecting cattle against bovine pasteurellosis described under sub-heads (*a*) to (*h*) indicated that, with the possible exception of heat-treated vaccine, none approximates in immunising value to the new formalinised vaccine the preparation of which in Roux flasks has been described. Accordingly, this vaccine was put into full-scale production for use in the Kenya field and the early success achieved with the vaccine in quelling outbreaks of an "explosive" character soon created a situation in which production could not keep pace with demand. The method of production was therefore altered as follows:—

(*a*) *Medium and sterilisation*: Ten-litre capacity sterile vaccine flasks are half filled with bovine meat-extract broth at *pH* 7.6 and 0.1 per cent hæmolysed bovine blood added to each flask. This enriching substance is prepared by adding one volume of freshly-drawn blood to 10 volumes of distilled water and filtering through a Seitz E.K. disc. Flasks are then sterilised at 15 lb. pressure for 30 minutes and incubated for three days at 37.5° C. to check sterility before seeding.

(*b*) *Maintenance of cultures, and preparation of seed material*: The virulence of vaccine strains N.C.T.C. 3195, 4308, Soy 1, Percival and Stanley is maintained as previously stated.

To prepare seed material, blood agar cultures of each strain are separately subcultured in 50 ml. quantities of nutrient broth *pH* 7.6 which are then incubated

at 37.5° C. for 18 hours. Vaccine flasks are inoculated with seed material immediately they are removed from the incubator. Ten ml. seed material of each strain is inoculated separately in each flask, one flask to each strain, Flasks are shaken thoroughly and replaced in the incubator and shaken frequently during the first 24 hours' incubation, which extends for seven days. The growth in vaccine flasks is then uniformly turbid with a viscous, slimy deposit. At the end of the period of incubation the growth in each flask is examined for purity and the degree of encapsulation of organisms; a good field must show a minimum of 50 per cent of organisms richly encapsulated. Formalin is then added to each flask to give a final concentration of 0.5 per cent.

(*c*) *Harvesting of vaccine*: The contents of vaccine flasks are now run through a Sharple's centrifuge operating at 23,000 revolutions per minute. A fine jet is necessary to effect complete clearance of bacterial growth. The deposit within the cylinders is emulsified with saline in an "Ato-Mix" emulsifier and the emulsion is adjusted to opacity tube 5 with 0.5 per cent formol saline. The finished product is then stored at 37.5° C. for three days.

(*d*) *Biological test*: Ten mice are given 0.25 ml. of the vaccine subcutaneously and challenged three weeks later with 100 LD_{50} (mouse) strain N.C.T.C. given intraperitoneally. Survival after challenge must be 90-100 per cent with 100 per cent death in 10 control mice.

The yield of vaccine by this method of production is low and averages 400 cattle doses (each 5.0 ml.) per 10 litres medium. The search for a growth factor to increase the harvest gave no positive results. The addition of iron filings to the medium to give partial anærobic conditions of growth did not succeed nor did the addition of glucose in concentrations from 0.1 to 1.0 per cent, nor sodium sulphite (0.025 per cent), nor liver extract, nor recrystallised chemically pure hæmin in trace quantities. Yet if the various strains of pasteurella used in the vaccine be grown in the standard blood broth sterilised by filtration (Seitz E.K.) growth is more prolific than in autoclaved blood broth. It would appear possible that adjuvant factors are present in blood broth that are destroyed by heat. We have been unable to utilise this finding in the mass production of the vaccine since risks of contamination are too great. The brand of peptone used in the preparation of the vaccine is immaterial. We have used several brands and found them all of equal value.

The following findings seem of importance in the production of the vaccine:—

(a) Incubation of flasks at 37.5° C. for a period of seven days not only gives more luxuriant growth than incubation of 18 to 24 hours but organisms are better encapsulated after prolonged incubation.

(b) Culture flasks, half filled with medium and frequently ærated by vigorous shaking during the first 24 hours of incubation give a better yield of organisms than do culture flasks filled to capacity and left unshaken in the incubator.

(c) It is a decided advantage to seed flasks when the medium is at a temperature of 37.5° C. This enables the organisms to gain a rapid start in growth.

(d) It is not necessary to freeze-dry cultures of pasteurella maintained for the preparation of the vaccine. Full virulence of the strains has been conserved over a period of three years by the technique described in the maintenance of cultures.

The following experiment shows that the keeping quality of the new vaccine is good even under adverse conditions of storage. A bottle of a routine batch of vaccine was kept for seven months on a laboratory bench at Kabete. At the end of this period 20 mice were each vaccinated with 0.25 ml. of the sample and challenged after one month with the standard challenge; 75 per cent vaccinated mice were solidly immune to the challenge which killed 10 mice used as controls to the experiment.

The new vaccine has been used extensively on Kenya cattle farms during the last three years. There have been numerous reports of its efficiency even to the point of stopping outbreaks, of an " explosive " character which formerly, we know, smouldered for months before the new vaccine was available. Bovine pasteurellosis has not appeared in any herd in which vaccination is practised at six-monthly intervals or even in herds where this period is extended to one year. We believe that immunity following vaccination is reasonably effective at one year but exercise caution by recommending vaccination every six months since the duration of immunity in cattle given the vaccine has not been determined. Table 5 shows that cattle can be immunised effectively for up to one year by the formalinised vaccine prepared from Roux flasks. On the other hand, outbreaks of the disease are still encountered in non-vaccinated herds and the incidence of the disease in them is, to the best of our knowledge, no less than during the year preceding the field issue of the new vaccine.

Summary

A brief account is given of the history and incidence of bovine pasteurellosis in Kenya. Strains of *Past. boviseptica* isolated from outbreaks in Kenya conform in general bacteriological details with one another and with two strains obtained from the National Collection of Type Cultures. Sera produced from rabbits by multi-inoculation with antigens variously prepared from Kenya and N.C.T.C. strains were of no value in typing these strains by the agglutination technique. Such strains were shown to be immunologically similar on the basis of cross protection tests in laboratory animals. The viability of these strains under varying conditions was determined as was their capacity to undergo bacterial mutation in differing cultural environment. The comparative virulence of strains of Kenya and N.C.T.C. origin was compared both in cattle and in laboratory animals. These strains were found to be generally resistant, on *in vitro* tests, to a wide range of drugs of the sulphonamide series, but sensitive to penicillin sodium salt G, Streptomycin and Aureomycin. However, *in vivo* tests in rabbits given a small infective dose of virulent culture of a selected strain showed the penicillin salt to be without effect in controlling the experimental disease.

Experiments in immunology showed that rabbits may be highly immunised by heat-treated vaccine prepared from *Past. boviseptica* strains selected through virtue of virulence and rich capsular content, and the same vaccine was found

to have a high protective value in cattle challenged subsequently by virulent culture which was lethal in controls. A formalinised broth culture vaccine in use in Kenya was found to be of no value, on standard dosage, in the protection of cattle and laboratory animals when challenged later with virulent culture. A concentrated formalinised vaccine, prepared from strains of high virulence and of rich capsular content was found to confer a high order of immunity both in cattle and laboratory animals. Fully susceptible cattle could be protected against a virulent infection, that killed all controls, up to a period of one year following vaccination. Particulars of this vaccine, its method of preparation and the biological standards prescribed for its use in the field are described.

Experiments were conducted to investigate other methods of prophylaxis against bovine pasteurellosis, viz., chick embryo vaccine, aggressin, immune serum, filtrate of broth culture, and saponin vaccine. Chick embryo vaccine and aggressin were of limited value and did not compare in immunising value with the new concentrated formalinised vaccine. Immune serum was of doubtful value as a prophylactic in cattle and laboratory animals. Saponin vaccine had no value.

Early success attended a vaccine prepared from a rough avirulent strain of *Past. boviseptica* of Kenya origin and a high degree of immunity could be conferred in cattle and rabbits with this vaccine. It was not possible to maintain the avirulent rough characters of this strain through successive sub-cultures. If this could be done, the experiments undertaken indicate that such a vaccine would be of the greatest value in the protection of cattle against bovine pasteurellosis.

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CRITICAL TESTS OF PIPERAZINE ADIPATE AS AN EQUINE ANTHELMINTIC

By T. E. GIBSON, M.V.Sc., M.R.C.V.S.
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A NUMBER of authors have tested the value of various piperazine compounds for the elimination of ascarid and strongylid infestation in the horse. Most workers have relied on the comparison of pre-treatment with post-treatment egg counts in assessing the value of the compounds tested. Relatively few critical tests have been carried out. When two horses carrying mixed infestations of *Trichonema* spp. and *Triodontophorus* spp. became available, it was decided to use them for critical tests with piperazine adipate. The results are recorded in this paper.

Materials and Methods

The animals used in this experiment were two foals which were born in April, 1955, and were kept in loose boxes with their dams. A strict regimen was employed which ensured that they remained free from nematode infestation until they were each given 30,000 strongylid larvæ on July 11, 1955. The foals were used then for certain observations which are not recorded in this paper, after which they were placed in separate loose boxes and used for critical tests of piperazine.

Fæcal egg counts were carried out three times weekly for a period of two weeks before the animals were treated with piperazine and then daily on the day of treatment and the four subsequent days. The foals were given the piperazine compound in water by stomach tube; pony 322 received 20 g. and pony 333 15 g., the dosage rate being 10 g. per 100 lb. body weight. After treatment all bedding was removed from the loose boxes so that all the fæces passed subsequent to treatment could be collected for examination. The fæces were collected for four days, the amount passed each 24-hour period being collected separately. Examination of the total fæcal output from each horse would have proved too time consuming, so a sample was taken from the fæces passed during each 24-hour period in the following manner. The whole sample was weighed and then broken up by rubbing between the hands. Then the whole sample was mixed thoroughly and divided into two, equal parts. Each half was treated separately. Each half sample was mixed again and then divided into two equal parts. One of these halves was discarded and the other subjected to further mixing. This procedure went on until the final sample weighed about 8 oz. The two 8-oz. samples were examined carefully for the presence of worms, a little at a time being placed in water in a petri dish over a black tile and examined by the aid of a lens. All the worms present were picked out separately and from the total present in each sample an estimate could be made of the total passed that day by simple calculation. The worms counted were cleared in lactophenol and identified.

Four days after treatment the foals were slaughtered and the cæcum and colon removed. These organs were slit longitudinally and the contents tipped out on to a table. The contents were weighed and a ½ lb. sample obtained by the

mixing process already described. The gut wall was placed on another table, and washed thoroughly under a strong jet of water. The washings were caught in a fine sieve. The contents of the sieve were examined carefully in a petri dish a little at a time, and the number of worms washed off the gut wall counted. The worms recovered were again cleared using lactophenol and identified.

Results

The results are summarised in Tables I and II. Table I gives the data on egg counts collected during the experiment and Table II records the results of

Table I

Date	Pony 332	Pony 333
7th November, 1955	500	400
9th " "	600	600
11th " "	700	300
14th " "	300	1100
16th " "	300	600
18th " "	200	600
21st " "	600	900
22nd " "	24	100
23rd " "	4	11
24th " "	1	6
25th " "	1	6

Ponies dosed with piperazine adipate 21st November.
Ponies killed for post-mortem examination 25th November.
Showing the strongylid egg counts of ponies 332 and 333.

Table II

	Pony 332			Pony 333		
	Worms expelled during 96 hours after dosing.	Worms remaining at post-mortem	Percentage efficiency	Worms expelled during 96 hours after dosing.	Worms remaining at post-mortem	Percentage efficiency
Trichonema spp.	670	33	95%	1458	75	95%
Triodontophorus spp.	306	96	76%	251	241	51%
Oxyuris equi larvae	277	2732	9%	4	75	0.5%
Oxyuris equi mature	51	130	28%	0	11	0%

Table II showing details of the critical tests on horses 332 and 333.

the worm counts and the percentage efficiency of the drug against the different species of worm present.

Although the details have not been given in the tables, it was found that no worms were expelled during period 72 hours to 96 hours following dosing, and very few during the previous 24-hour period. No appreciable loss of accuracy

would have occurred if the fæces had been collected for only 48 hours following dosing.

Discussion

The results obtained in this experiment are in general agreement with the work of others who have carried out critical tests. In the present work 95 per cent efficiency was obtained in the two horses against *Trichonema* spp., a result in close agreement with Poynter (1955), who recorded 100 per cent efficiency against "small strongyles," and with Downing *et al.* (1955), who recorded virtually 100 per cent efficiency against *Trichonema* spp. In the present work variable efficiency was obtained against *Triodontophorus* spp., namely 76 per cent in one animal and 55 per cent in the other; a finding similar to that of Downing *et al.* (1955). The action against *Oxyuris equi* was poor.

Unfortunately the horses used in the present work were not carrying any worms of *Strongylus* spp., but Downing *et al.* (1955) have recorded poor efficiency against these species. It is clear, therefore, that the action of piperazine adipate against strongylids in the horse is very similar to that of phenothiazine, being very efficient against the small strongyles and indifferent against *Strongylus* spp. As well as possessing an efficiency against strongylids equal to that of phenothiazine, piperazine adipate has the additional advantage of being an efficient ascaricide and of being considerably less toxic. It seems that piperazine compounds have an advantage over phenothiazine as an anthelmintic for the horse.

Summary

Two critical tests of piperazine adipate on horses showed that at a dosage rate of 10 g. per 100 lb. bodyweight the drug is 95 per cent efficient in eliminating *Trichonema* spp., over 50 per cent efficient against *Triodontophorus* spp., but is of little value against *Oxyuris equi*.

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REPORT

REPORT ON THE ANIMAL HEALTH SERVICES IN GREAT BRITAIN. 1955. Ministry of Agriculture, Fisheries and Food; Department of Agriculture for Scotland. H.M.S.O. 1956. 5s.

THIS report includes sections on animal diseases, regulations which have been designed for the prevention of the introduction and spread of disease, the protection of animals during transit, the export of livestock and their products, and the research and investigation services. The appendices contain tables which show the geographical distribution of the diseases under review, the numbers of animals imported into Great Britain and the incidence of some of the contagious diseases in European countries; other tables demonstrate the progress made in the eradication of bovine tuberculosis.

With the exception of 1930, there were fewer outbreaks of foot-and-mouth disease in Great Britain in 1955 than in any year since 1918. Nine only were reported and types "C" and "O" were identified. Some of the recommendations of the Departmental Committee on Foot-and-Mouth Disease 1952 to 1954 are now adopted; the standard size of an infected area around an outbreak has been reduced from a radius of 15 miles to one of 10 miles, and the practice of salvaging for human consumption the carcases of apparently healthy animals slaughtered as contacts, has been discontinued.

Slightly less swine fever occurred in 1955 than in 1954, and there was no variation of any consequence in the demand for swine fever (crystal violet) vaccine. Atrophic rhinitis was observed in eight herds of pigs, and all were Landrace pigs or pigs which had been reared in contact with them.

Apart from 1952, when 1,215 outbreaks of anthrax were recorded, 1955 provided the highest total for many years. The officers in the field suggest that feeding stuffs were probably the source of infection in 592 of the 764 outbreaks; feeding stuffs and fertilisers were suspected in 70 further outbreaks and fertilisers alone in another 16. Cattle were the most common species to succumb, with pigs and mink next in order. The report emphasises the danger of cutting carcases and shedding blood in suspected cases; the prohibition of this activity is also mentioned.

Fowl pest continues to exact a heavy toll and nearly half a million pounds was paid in compensation in 1955 consequent upon 906 outbreaks. A very high number of the outbreaks were secondary ones and the spread of infection seems to be associated with the activities of a comparatively small number of poultry dealers and slaughterers who handled live or dead poultry in the process of distribution.

"In the 20 years of work under the Attested Herds Schemes, the stage has been reached when nearly two-thirds of the cattle in the country are in Attested Herds, and there is every reason to hope that by the early 1960's bovine tuberculosis in Great Britain will, for all practical purposes, be a thing of the past." This excerpt from the report is taken from the first paragraph of the section

dealing with bovine tuberculosis. The progress made is appreciated when it is realised that in 1931 about 40 per cent of cows were probably infected with tuberculosis; this would represent an incidence of something over 25 per cent of all cattle. In 1935 about 5,000 cattle were in attested herds and attested areas; in 1945 this figure was over 788,000, and in 1955 over 6,000,000; the latter figure represents 62 per cent of all cattle. These figures are related to the number of badly infected cattle slaughtered under the Tuberculosis Order of 1938, which has dropped from the pre-war level of 20,000 a year to about 1,250; of the milk sold in Great Britain, 75 per cent is produced from herds which are regularly tuberculin tested, as compared with 42 per cent five years ago and only 15 per cent ten years ago.

Nearly 2,000 dogs and over 200 cats were landed in Great Britain during 1955 under the Importation of Dogs and Cats Order of 1928; the order is designed to prevent the introduction of rabies, and 12 persons were convicted during the year for contraventions of the Order. The importation of semen derived from cattle, horses, pigs, sheep and goats is also controlled.

Some countries prohibit the importation of animals from Great Britain as a safeguard against the introduction of certain diseases. Swine fever in pigs and scrapie in sheep are feared by Australia; Canada and New Zealand continued to prohibit the importation of sheep because of the possible danger of scrapie, and New Zealand placed a temporary ban on pigs owing to outbreaks of atrophic rhinitis in this country.

The section concerned with research and investigation covers a wide field and includes work on Brucella and Vibrio infections, Anthrax, Johne's disease, Tuberculosis and Mastitis. The value of a vaccine against Johne's disease is being judged on a comparison of the incidence of the disease in a herd before and at least seven years after vaccination. The results over a period of nine years in 55 infected herds have been excellent. Some attenuated strains of myxomatosis have been encountered, and rabbits inoculated with these strains develop a nodular type of disease and recover; material collected from œdematous nodules during the early stages of the disease contains virulent virus; that collected from the shrunken dry nodules in the later stages contains attenuated virus. Two cases of myxomatosis in hares were confirmed. Parasitological and mycological investigations are also proceeding, as well as work on metabolic disorders, diseases of poultry and the problems which arise in connection with artificial insemination.

The statistical tables and a list of publications by members of the staff conclude a report which is a creditable record of services rendered; these services aid the economic well-being of Great Britain and play their part in increasing our knowledge of some of the evils which beset the animal world.

XUM

REVIEWS

VETERINARY CLINICAL DIAGNOSIS, by Prof. D. Wirth. London: Baillière, Tindall & Cox Ltd. Pp. 230 + 213 illust. Price 25s.

A GOOD clinician needs the eyes of a seer, the hands of a virtuoso and the judgment of a Solomon. When he possesses these attributes in fair measure the animal body becomes transparent and the manifestations of aberrations of structure and function can be recognised with relative ease. Deviations from the normal are reflected in changes in structure and/or function in other parts of the body and an observant man reasonably versed in a knowledge of anatomy and physiology can, by taking account of such changes, deduce the site and probable nature of the primary lesion.

In these days there are so many ancillary aids to diagnosis that a bundle of specialised reports may be furnished for each patient and a clerk without medical knowledge could make a diagnosis on the evidence presented and without seeing the patient. One has to be careful that one's eyes and hands do not lose their cunning. There is a danger that the clinical acumen so well developed in previous generations may be lost for ever.

Professor Wirth's book has been made available to the English-speaking people at an opportune time. Many young veterinary surgeons will benefit greatly from almost every line of this book. It will intrigue them and it will educate them to recognise the portent of the obvious signs and symptoms which are presented before their eyes by each patient.

The illustrations are numerous and so good that if they were collected together they, in themselves, would make an excellent "Aid to Clinical Diagnosis."

LABORATORY CONTROL OF DAIRY PLANT, by J. G. Davis, D.Sc., Ph.D., F.R.I.C. London: Dairy Industries Ltd., 1956. Pp. 395. Numerous tables, figures and references. Price 30s.

TO-DAY milk is subjected to many extensive processes; milk is produced, handled, cooled, pasteurised or sterilised, bottled and distributed. The equipment necessary for these operations has to be cleaned, sterilised and maintained; the efficiency of the operations must be checked constantly and the knowledge which tells one whether the required standards have been reached can only be obtained by laboratory tests. Many of the standards are contained in "The Milk and Dairies Regulations, 1949, and the "Milk (Special Designation) (Pasteurised and Sterilised Milk) Regulations, 1949" Acts. Additional equipment is necessary when milk is manufactured into cheese or butter, and when it is powdered, condensed or evaporated. Collecting depôts, pasteurising depôts and manufacturing depôts play their part in the dairy industry and the laboratory is essential to them all. The laboratory is also necessary for factories dealing with drinks and foods of other descriptions and this volume may have some application to these industries.

Chapter headings give an indication of the scope of the book. There are 18 of them and they are concerned with the plant and the materials used which include water, detergents, sterilants, bottles, effluents and refrigerants. Con-

trolling instruments in the dairy plant and corrosion and trace metal problems also have a place. Economics and legal aspects are considered and there are 19 pages of references. There are many pictures, figures and tables and one can find a wide field of information bearing upon such problems as the weight of a milk bottle, the analysis of detergent mixtures, the bacteriological examination of water, the cost of steam-raising by solid fuel and the factors which control boiler efficiency.

The object of this book is to enable dairy managers and others to attain a high degree of efficiency in the management of a modern dairy. It will go a long way to ensure this objective.

NOTICES

Imperial Chemical (Pharmaceuticals) Limited

For convenience of internal administration, the business and assets of Imperial Chemical (Pharmaceuticals) Limited have been transferred to Imperial Chemical Industries Limited Pharmaceuticals Division, and as from January 1, 1957, the business of I.C.(P) will be carried on by that Division.

There will be no change in the methods by which I.C.(P)'s business has been conducted, in the range of products at present sold, or in the staff.

Milton-Deosan (Scotland) Limited

In conjunction with their Scottish Agents, Messrs. Thomas Henderson & Co. Ltd., Milton Antiseptic Ltd. announce that the above company was incorporated on February 1, 1957, registered in Scotland. This step is being taken to facilitate the administration of Milton-Deosan interests in Scotland.

Burroughs Wellcome & Co. announce that "Wellcome" brand Feline Infectious Enteritis Vaccine is now issued under the trade mark "Fiovax," in a pack containing 2 x 1 c.c. vials.

OBITUARY

Death of Vice-Chairman of the Crookes Laboratories

Vice-chairman of The Crookes Laboratories, Park Royal, London, since November, 1932, Mr. William Jessup Wilson (aged 76) died suddenly after a short illness at his home on January 30.

Publishers' Notices

The British Veterinary Journal, with which is incorporated The Veterinary Journal, is published monthly, and copy for advertisements should be in the hands of the advertisement manager not later than the 20th of the preceding month, if proof is required. Tel.: Temple Bar 3386.

Letters for the Journal, literary contributions, reports, notices, books for review, exchanges, new instruments or materials, and all matter for publication (except advertisements) should be addressed to the Editor.

Annual Subscription, 40s. ($6.50 U.S.A. currency, post free).